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EDITOR'S NOTE

Line 7 on p. 184 of the article by Merkel H. Jacobs should read:

$$\dot{\Delta} = 1.86C + 0.2C^2,$$

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE

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HYPOPHYSIAL CONTROL OF CUTANEOUS PIGMENTATION IN AN ELASMOBRANCH FISH[1]

HELEN M. LUNDSTROM AND PHILIP BARD

EDITOR'S NOTE:

The equation on page 277 of the article by E. N. Harvey in the December, 1931, issue should be corrected to read as follows:

$$\sigma = \frac{2.12 \times 10^{-7} \times .8 \times 10^{-2} \times 10^{3} \times 1.6 \times 10^{3}}{6.28 \times 23 \times 10^{-4}} = 0.19 \text{ dynes per cm.}$$

P. E. Smith (1916), Allen (1917, 1920), Atwell (1919), Rehberg and Krogh (Krogh, 1922) and Hogben and his collaborators (Hogben, 1924). It is a curious fact that, so far as we have been able to ascertain, no experimental investigation of chromatic function in the elasmobranch group has ever been made, although these animals possess dermal chromatophores (melanophores and xanthophores) not unlike those seen in the frog (Daniel, 1922).

That pituitary removal alone is followed by the development of pallor was shown in a series of hypophysectomies performed on 40 dogfish. The operation is easily carried out through a buccal approach.

---

[1] A report of results obtained in an investigation carried out in the Course in Physiology at the Marine Biological Laboratory, Woods Hole, during the summer of 1931.

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HYPOPHYSIAL CONTROL OF CUTANEOUS PIGMENTATION IN AN ELASMOBRANCH FISH[1]

HELEN M. LUNDSTROM AND PHILIP BARD

(*From the Marine Biological Laboratory, Woods Hole*)

In a study of the effects of ablation of various parts of the brain of the dogfish, *Mustelis canis*, it was noticed that certain operated individuals became, in the course of the first few post-operative hours, lighter in color than their fellows. The degree of paling was such as to render the affected animal distinctly lighter than any normal dogfish of this species that we have seen. Inspection of the cranial contents of the operated fish showed that whenever the albinous appearance was produced the pituitary body had been extirpated in the intracranial intervention. On the other hand, the hypophysis was invariably intact in those operated fish which retained their normal dark grayish color. Quite naturally we were led to suspect that the dogfish possesses an hypophysial control of its cutaneous pigmentation similar to that which has been so clearly established for the amphibia by P. E. Smith (1916), Allen (1917, 1920), Atwell (1919), Rehberg and Krogh (Krogh, 1922) and Hogben and his collaborators (Hogben, 1924). It is a curious fact that, so far as we have been able to ascertain, no experimental investigation of chromatic function in the elasmobranch group has ever been made, although these animals possess dermal chromatophores (melanophores and xanthophores) not unlike those seen in the frog (Daniel, 1922).

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[1] A report of results obtained in an investigation carried out in the Course in Physiology at the Marine Biological Laboratory, Woods Hole, during the summer of 1931.

Method

The fish was tied down in the dorsal position on a small shark board and a stream of sea water introduced by means of a tube into the pharyngeal region to maintain respiration (under these circumstances normal breathing movements continue). After incision and retraction of the mucous membrane the base of the skull was lightly scraped along the midline at the level of the pupils of the eyes. This brings to view through the translucent cartilage the chiasma, hypothalami, and posterior hypophysis. A medial opening was then cut in the skull between the chiasma and the posterior lobe. This exposes the elongated anterior lobe of the pituitary which lies in the depression between the hypothalami and extends forward nearly to the chiasma. The width of the exposure was such as to lay bare the medial half of each ventral hypothalamic surface. So much may be done without the slightest hemorrhage. But it was found that extension of the skull defect backward so as to expose merely the cranial aspect of the large bifid neuro-intermediate or posterior lobe resulted in profuse bleeding. Therefore this structure was carefully scooped out by means of a small curette introduced through the limited opening. With a little care this can be done without provoking hemorrhage and with minimal injury to the base of the brain. The anterior lobe may be left intact or it may be picked up with forceps, separated from the overlying nervous tissue, and so extirpated with very little damage to the hypothalamus. No attempt was made to close the wound. Provided the base of the brain had not been severely traumatized, the operated fish swam around normally and showed a general behavior indistinguishable from that of unoperated specimens. They usually succumbed after three or four days with loss of righting reactions and dyspnea as premonitory symptoms. A few lived as long as a week, which is about the average length of life of unoperated specimens kept under laboratory conditions. It seems likely that death was often induced by cerebral injury, some degree of cerebral herniation being the usual finding at autopsy. We have not been disturbed by this rather crude postoperative situation. It is apparently without significance for the specific problem in hand, namely, the experimental analysis of pituitary control of cutaneous pigmentation, for injury or herniation of the hypothalamic region without removal of the hypophysis never gave rise to the slightest pallor. Several animals survived such a condition several days and retained the same shade as normal control animals.

RESULTS

Complete removal of the pituitary body (30 experiments) always resulted in pallor. Slight paling was first noticeable about thirty minutes after pituitary ablation and after three hours the change was generally very marked, but maximal pallor was not attained until about the twelfth postoperative hour. It was our practice to select pairs of fish having the same degree of pigmentation, hypophysectomize one and retain the other for comparison. Plate 1 is a photograph of two such fish taken twenty-four hours after complete hypophysectomy in one. It shows clearly the extreme pallor characteristic of the animal without hypophysis. That the pallor is due to "contraction" of the dermal melanophores is shown in Plates 3 and 4, which are microphotographs of homologous pieces of skin taken from the same fish just before and nineteen hours after hypophysectomy. As shown in Plate 3 the melanophores of the normal fish are in a state of considerable expansion, while Plate 4 indicates clearly that the pallor developing after pituitary removal is the result of the assumption by the melanophores of a more or less rounded contour. Were it not for the outlines of several placoid scales one might easily mistake these plates for microphotographs of the skin of a normal and a hypophysectomized frog or axolotl.

Removal of the anterior lobe alone was done in five dogfish and these survived the operation for several days without showing the slightest change in cutaneous pigmentation. Nor did leaving the anterior lobe intact impede in any way the development of the pallor ensuing upon removal of the posterior lobe. As already mentioned, severe traumatization of the hypothalamus was found to be without influence on pigmentation provided the posterior lobe of the pituitary was not ablated. In four fish the anterior lobe and hypothalamic protuberances were exposed in the usual way, transverse incisions made in these structures to a depth of one or two millimeters and much of the substance of the hypothalamus pulled out with forceps. Two of these animals survived thirty hours without at any time presenting the slightest change in hue. The other two, whose color also remained unchanged, were subjected to posterior pituitary removal after twenty-four hours whereupon there ensued a pallor which developed at the usual rate and to the usual extent.

Further evidence for an hypophysial control of the cutaneous melanophores was sought in a study of the effects of pituitary preparations when injected into pale hypophysectomized dogfish. Each of five pale specimens received subcutaneously a crushed dogfish posterior lobe suspended in one or two cubic centimeters of sea water. A distinct general darkening appeared within the first three minutes and after an hour these fish were indistinguishable from the darkest normal animals. Then in the course of the next five or six hours they gradually returned to the albinous condition. Injections of similar suspensions of cerebellum, skeletal muscle, and pancreas from normal dogfish were entirely without effect. In three experiments it was found that administration of crushed anterior lobe resulted in a moderate darkening which was slower to develop and which disappeared more rapidly than the more intense darkening caused by an equal quantity of crushed posterior lobe. In one pair of pale fish injection into one of a crushed posterior lobe fragment of about half the size of the anterior lobe produced an immediate darkening which reached its maximum in one hour, while injection of the crushed anterior lobes of two fish into the other produced a moderate darkening which was slow to develop and had a very short duration. These results together with those of the ablation experiments indicate that the posterior lobe is the chief source of the melanophore-expanding substance or secretion. The activity of the anterior lobe material may well have been due to diffusion into it of active substance of posterior lobe origin. Shortage of fish prevented our exploring this question further.

Extracts of several posterior lobes were made. Immediately after its removal from a living dogfish the lobe was crushed, ground up, stirred and shaken in sea water, and the resulting suspension filtered. After appropriate dilution with sea water the clear filtrate was tested by injection of varying amounts into thoroughly pale hypophysectomized fish of the same size as the donor of the lobe. It was found that an amount of filtrate corresponding to one twenty-fifth of one posterior lobe was necessary to produce a noticeable darkening while it required from one-tenth to one-seventh of a filtrate to make such a fish as dark as normal controls. It is unlikely that such simple extracts as these represent the full potency of the glands. Hogben (1924) states that the amount of melanophore stimulant in the pituitary of one frog is sufficient to darken the skin of fifty-six animals of the same species.

The posterior pituitary preparations "pituitrin" (Parke, Davis & Co.) and "infundin" (Burroughs, Wellcome & Co.) produced darkening in pale pituitaryless fish when given subcutaneously or intramuscularly. Plate 2 is a photograph of the pair of fish shown in Plate 1. It was taken one hour after the hypophysectomized individual had received a subcutaneous injection of 0.4 cc. of "obstetrical pituitrin," an amount equivalent to four international oxytocic units. It can be seen that this dose had darkened the animal almost to its preoperative shade. In the case of several pale hypophysectomized fish, twenty to thirty inches in length, it was found that a subcutaneous dose of about 0.5 cc. (5 international units) of "obstetrical pituitrin" was required to produce in them a darkness equal to that of normal controls. It seemed interesting to test the action on elasmobranch melanophores of the two active principles separated from pituitary extracts by Kamm and his associates and now supplied by Parke, Davis and Co. under the names "pitressin" and "pitocin." It was found that 0.25 cc. (5 pressor units) of "pitressin" was capable of producing approximately the same effect as 0.5 cc. (5 international units) of "pituitrin." On the other hand 0.5 cc. (5 international units) of "pitocin" had no effect whatever on pale individuals of the same size. When twice this quantity of "pitocin" was injected there occurred a slight darkening which reached its maximum much more slowly and disappeared more rapidly than the reaction to "pitressin" or "pituitrin." Since "pitocin" contains one-half unit of pressor activity per cubic centimeter, it is probable that this mild positive result was due to the pressor substance present. These results are in general agreement with those of L. W. Rowe (1928), who reported that "vasopressin" ("pitressin") but not "oxytocin" ("pitocin") caused expansion of frog melanophores, a finding which is in contradiction to the inference of Hogben and Winton (1922*b*) that the uterine and melanophore stimulants of pituitary extracts are identical.

We had hoped to secure evidence of the presence of melanophore-expanding material in the circulating blood, but shortage of dogfish during the latter part of the summer made this impossible. Indirect evidence that pituitary substance acts directly on the dermal melanophores was, however, secured in our observation, often repeated, that the melanophores of small isolated pieces of skin from pale dogfish expanded markedly when such skin fragments were placed in dilute sea-water solutions of "pituitrin" or "pitressin" or in suspensions or extracts of dogfish posterior lobe. When such pieces were immersed in sea water alone the melanophores remained contracted. The expanded melanophores of bits of skin from normal fish gradually contract when placed in sea water. Addition of "pituitrin," etc., prevented this slow contraction. Equivalent solutions of "pitocin" had no action on the melanophores of skin fragments.

Discussion

The results presented in the foregoing section give abundant proof that the normal dark grayish color of the dogfish is maintained through the agency of the hypophysis. Apparently this organ continuously delivers to the blood stream a quantity of melanophore stimulant capable of maintaining the rather widely expanded state of the cutaneous melanophores to which the normal color of the fish is due. The experimental findings strongly suggest that this humoral agent has its chief, if not its sole, origin in the posterior lobe. This portion of the gland is more correctly termed the neuro-intermediate lobe. Apart from a rather small extension of neuroglia fibers from the border of the infundibular cavity it is wholly composed of a highly vascular mass of basophil cells which, on the basis of embryological as well as histological observations, obviously constitutes the homologue of the pars intermedia of higher forms (de Beer, 1926). The ventral lobe of the selachian hypophysis which is attached dorsally to the neuro-intermediate lobe and ventrally to the skull and which, according to de Beer (1926), is composed largely but not entirely of basophil cells, has not been taken into separate account in our experiments. In all of our posterior lobe extirpations it was doubtless removed. It would seem highly probable that the melanophore-expanding substance originates in the pars intermedia. There is excellent evidence that such is the case in the amphibia. We know that there too anterior lobe removal is not followed by pallor (Hogben and Winton, 1923) and that while the melanophore stimulant is not lacking from extracts of pars anterior and pars nervosa of beef glands, there is more of it per gram of dried tissue in the pars intermedia than in the other two portions of the gland (Hogben and Winton, 1922*a*, 1922*b*). Some further confirmation of this point comes from Atwell's report (1919) that albinous tadpoles darken as a result of melanophore expansion when placed in dilute extracts of beef pars intermedia. But by far the most conclusive results have been those obtained by B. M. Allen (1920) in his study of the effects of transplantation of different parts of the hypophysis of the adult frog (*Rana pipiens*) into tadpoles from which the pars buccalis had been removed (anlage of pars nervosa left intact) and which, as a result, exhibited pallor as well as failure to metamorphose and retardation of growth. The operated larvae which received anterior lobe transplants showed "not the slightest tendency to return to the original black color except for a slight tendency at the beginning," although this treatment caused marked acceleration of growth. On the other hand, the albinous tadpoles regained their dark color when the intermediate lobe was engrafted into them. In a recent communication (1930) Allen reports experiments in confirmation of his earlier results and shows that transplants of pars nervosa do not cause pigmentary effects. Allen's work is a most significant contribution to the problem of localization of pituitary function. Although it presents the only unequivocal evidence for the precise origin of the amphibian melanophore stimulant, it is not referred to in Hogben's monograph (1924). Shortly after the appearance of Allen's paper, Swingle (1921) reported that intraperitoneal transplantations of pars intermedia from various frogs into bullfrog larvæ caused darkening of the skin. It can be concluded that in the frog the pigmentary hormone is produced only by the pars intermedia and our own findings lend support to the view that such is the case in the elasmobranch.

It is well known that amphibia exhibit a rhythm of color response which depends on a balance of such environmental factors as humidity, temperature, oxygen supply, and illumination (Hogben, 1924). In the majority of amphibia light causes pallor while its absence promotes darkening. We have found that dogfish taken from a moderately illuminated tank paled markedly when placed in a brightly illuminated tank, while other fish originally of the same shade and from the same tank darkened perceptibly when placed in a tank from which nearly all light was excluded. These changes require several hours. They cannot, of course, be referred to changes in humidity and we were at some pains to be sure that during these tests the temperature and aëration of the tanks were equal. Segments of illuminated fish which were covered by bands of adhesive plaster did not remain dark. We conclude, therefore, that light causes pallor by a general rather than a local action, and since pale hypophysectomized fish remained pale when kept in darkened tanks we can assume that these pigmentary changes evoked by changes in illumination are the results of variations in pituitary activity.

Hogben (1924) is strongly of the opinion that regulation of color response in the amphibia by melanophore contraction and expansion is correlated with a fluctuating pituitary secretion. He believes this to be an adequate explanation of all the pigmentary phenomena seen in these animals and he is inclined completely to discount all assumptions of a direct nervous control of amphibian melanophores. He suggests that such chromatic effects as have been observed following nerve section or stimulation or drug administration have been due to vasomotor changes. Since it is clearly established that adrenalin injected intravenously causes pallor in the frog we made some effort to determine whether injection of this substance has any influence on the pigmentation of the dogfish. In a few preliminary experiments we gained the impression that large subcutaneous injections of adrenalin caused slight paling. Subsequently, at a time when the supply of fish had greatly diminished, we tested the effect of injections made

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directly into the heart chambers. In collaboration with Dr. René Gayet, who kindly offered to share a number of fish with us, it was found that 1 cc. of a 1 : 1000 solution of adrenalin chloride evoked a paling which usually became noticeable after from five to ten minutes, sometimes not until after fifteen minutes, progressed to a maximum in about an hour, and persisted for approximately two hours. In one case the pallor was maximal; in the others it was not. Direct microscopical examination showed that in these cases the melanophores had actually undergone contraction. But the inadequate number of experiments performed makes it hazardous to venture an inference. Yet the comparative sluggishness of the responses provoked by these enormous doses which obviously produced a state of shock does not strongly suggest that the dogfish possesses an adrenal or sympathetic control. It may well have been that vasoconstriction of cutaneous vessels led to an asphyxiation and consequent contraction of the melanophores. Because of scarcity of material we were obliged to postpone to a later occasion an adequate investigation of the interesting question of a possible extra-pituitary control of elasmobranch chromatophores.

Summary

1. Complete hypophysectomy in the dogfish, *Mustelis canis*, results in pallor of the skin. This same result follows removal of the posterior (neuro-intermediate) lobe alone. Extirpation of the anterior lobe does not result in paling. Severe traumatization of the hypothalamus without destruction of the posterior lobe of the hypophysis has no influence on cutaneous pigmentation.

2. The pallor following hypophysectomy is due to "contraction" of the cutaneous melanophores which are normally in a state of considerable "expansion."

3. Suspensions or extracts of dogfish posterior lobe and the commercial posterior pituitary preparations, "pituitrin," "infundin," and "pitressin" produce melanophore expansion and consequent darkening when injected subcutaneously or intramuscularly into pale hypophysectomized dogfish. Suspensions of dogfish cerebellum, skeletal muscle, and pancreas have no effect upon pigmentation. The oxytocic principle of the preparation, "pitocin," does not produce melanophore expansion in hypophysectomized fish. Administration of suspensions of dogfish anterior lobe cause a relatively weak darkening of pale fish. The melanophores of isolated pieces of dogfish skin react to these various substances in the same way as do the melanophores of fish receiving them by injection.

4. Bright illumination of normal dogfish for several hours causes a

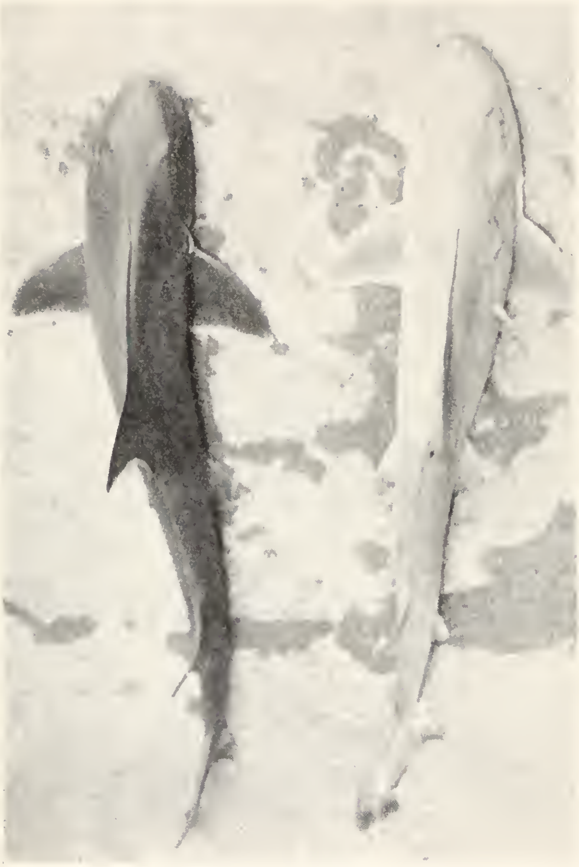

Plate 1. Photograph of a pair of dogfish, originally of the same shade, taken twenty-four hours after removal of the hypophysis in the animal on the right. Both animals had been kept in the same tank and the operated individual showed no abnormalities of behavior.

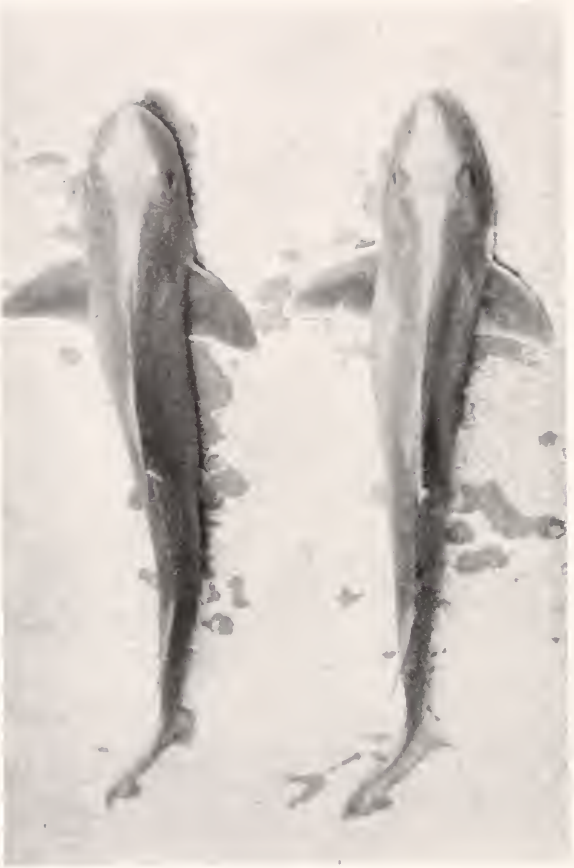

Plate 2. Photograph of the same pair of dogfish taken one hour after the hypophysectomized individual (on the right) had received a subcutaneous injection of 0.4 cc of "obstetrical pituitrin" (Parke, Davis & Co.).

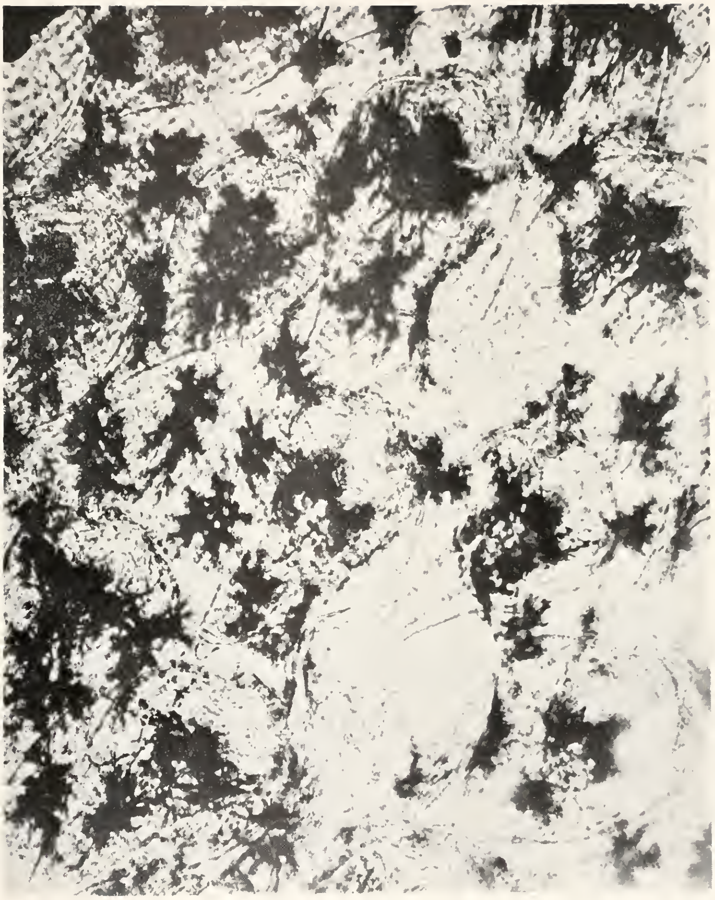

Plate 3. Microphotograph of a mount of skin removed from a normal dogfish showing expanded condition of the melanophores.

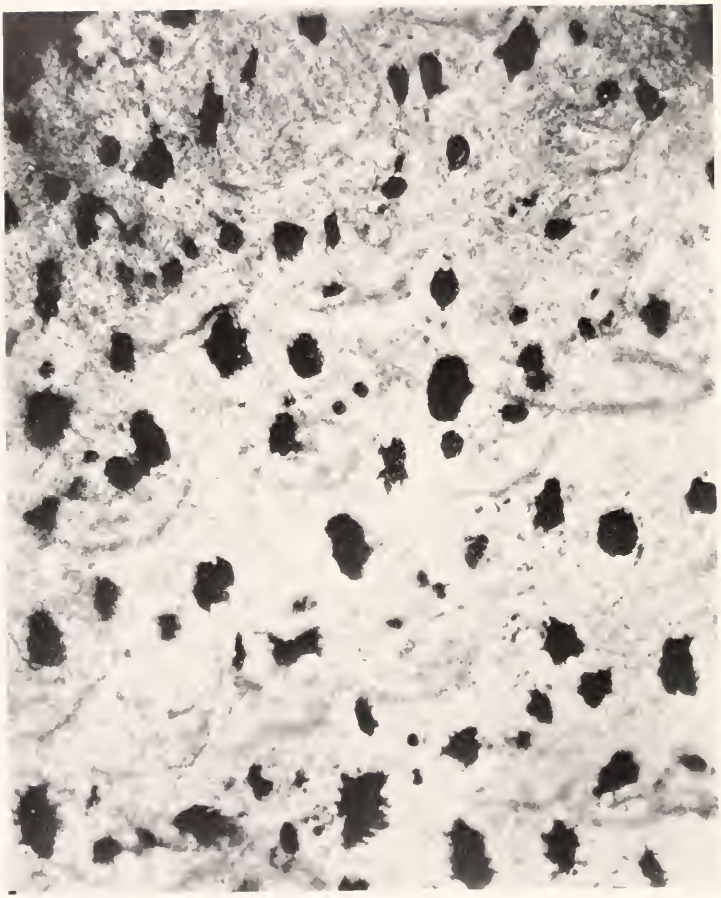

PLATE 4. Microphotograph of an homologous piece of skin taken from the same fish nineteen hours after hypophysectomy.

certain degree of pallor. Covered segments of the skin pale to the same extent as exposed regions. Absence of light leads to darkening of normal fish, but darkness does not modify the pallor of hypophysectomized individuals.

It is concluded that the posterior lobe of the hypophysis is responsible for the considerable degree of melanophore expansion characteristic of the skin of normal dogfish. Although the possibility of an extra-hypophysial control of cutaneous pigmentation has not been excluded, it is apparent that the pituitary gland plays a dominant rôle in the pigmentary alterations exhibited by the normal dogfish.

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REFLEX CARDIAC INHIBITION OF BRANCHIO-VASCULAR ORIGIN IN THE ELASMOBRANCH, SQUALUS ACANTHIAS

BRENTON R. LUTZ AND LELAND C. WYMAN

(*From the Mount Desert Island Biological Laboratory and the Physiological Laboratory of Boston University School of Medicine*)

Reflex cardiac and respiratory inhibition has been observed in elasmobranchs upon mechanical or electrical stimulation of various regions, external and internal, including the gill region (Lutz, 1930*a*). Reflex cardiac inhibition in mammals may also be obtained upon stimulating various regions. The distribution of the latter sensory areas is, however, much more restricted than in the elasmobranchs. Receptors which are especially important in regulating the circulation appear to be located in the vascular organs themselves. The depressor mechanism with receptors located in the aorta is well known (Eyster and Hooker, 1908; Anrep and Segall, 1926), and recently the carotid sinus has been shown to be an important zone, reflexly controlling heart rate and vasomotor tonus (Heymans, 1929). The physiological stimulus is apparently an alteration of pressure within this vessel.

The present paper is concerned with an attempt to ascertain whether similar alterations of pressure within the gill vessels of elasmobranchs are effective cardio-inhibitory stimuli.

MATERIAL AND METHOD

Specimens of the dogfish, *Squalus acanthias*, 600 to 1500 grams in weight, taken during the month of August from Frenchman Bay, Maine, were used. The spinal cord was pithed posteriorly from the level of the sixth vertebra. The fish was secured ventral side up in a shallow tank of sea water. Although respiration continued in an apparently normal way, perfusion of the gills with sea water through the mouth was carried out in most cases, in order to insure an adequate supply of oxygen. The ventral aorta was exposed and ligated between its first and second branches. The former give rise to the first and second afferent branchial arteries which supply the hemibranch and the first holobranch. Therefore the second, third and fourth holobranchs were functional. Changes of pressure in the first branches and their derivatives were effected through a cannula inserted in the ventral aorta, anterior to the ligature, and connected with a burette filled with a physiological solution (urea-saline, Lutz, 1930*b*). The heart beat was recorded on a smoked drum by means of a heart lever or a mercury manometer recording ventral aortic pressure through a cannula inserted posteriorly to the ligature.

RESULTS

Cardiac inhibition was obtained when the first ventral aortic branches, and thus the gill vessels arising from them, were suddenly subjected to increases of internal pressure applied by opening the connection between the cannula and the burette for varying periods (Fig. 1, A). The amount of fluid entering during the application of pressure varied from 0.2 cc. to 5 cc., depending upon the height at which the burette was set and the length of time it was held open.

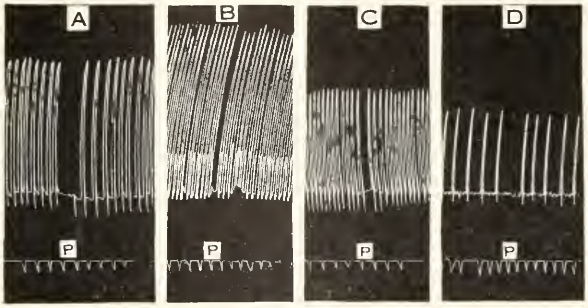

FIG. 1. Reflex cardiac inhibition following sudden increase of pressure within the gill vessels at *P*. Heart beat recorded by lever. In this and in subsequent figures the time record shows five-second intervals. Cord pithed posteriorly from the level of the sixth vertebra. Urea-saline solution in pressure burette except in B. A. Increase of pressure in the first branches of the ventral aorta. B. Same, with dogfish blood in the pressure burette. C. Same, with gill capillaries occluded with cornstarch so that no fluid entered. D. Increase of pressure in the third, fourth and fifth branchial arteries.

Inhibition was invariably obtained when the pressure applied (50 to 125 cm. of urea-saline) was obviously much higher than the dorsal aortic blood pressure. Simultaneous measurements of the ventral and dorsal aortic blood pressures showed that the pressure differences between the two aortae averaged 16.1 mm. Hg for systolic pressure and 4.3 mm. Hg for diastolic pressure, and that the pulse wave is transmitted through the gill capillaries to the dorsal aorta (Fig. 2, A). It is apparent, therefore, that the gill capillaries are relatively wide, and that the pressure in the afferent aortic system beyond the ligature is essentially the same as that in the dorsal aorta. Dorsal aortic systolic pressure ranged from 11 to 28 mm. Hg (8 measurements), and it was noted that fluid would not enter the ventral aorta from the burette below 25 cm. urea-saline (18.6 mm. Hg). A sudden increase of pressure averaging 10.7 mm. Hg above the average systolic pressure existing in the dorsal aorta was found to constitute an adequate stimulus for cardiac inhibition (Fig. 2, B), and in one instance a difference as low as 3 mm. Hg produced the response (Fig. 2, C).

Although urea-saline is considered to be a physiological solution, a control experiment was performed using heparinized fresh dogfish blood in the burette and its connections. A similar inhibitory response to increased pressure in the gill vessels was obtained (Fig. 1, B).

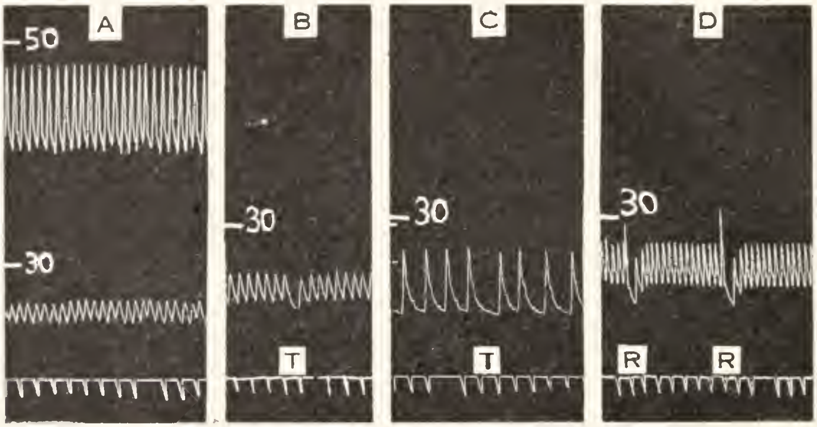

FIG. 2. Heart beat recorded by Hg manometer. Figures at left show pressure levels in mm. Hg. A. Simultaneous records of ventral (upper) and dorsal (lower) aortic blood pressure. B. Ventral aortic blood pressure record. Threshold increase of pressure in the first branches of the ventral aorta at *T* (33 cm. urea-saline, 24.6 mm. Hg). C. Same, threshold 25 cm. urea-saline (18.6 mm. Hg). D. Ventral aortic blood pressure record. Spontaneous ejection reflexes at *R*.

Cardiac inhibition was obtained when the burette was opened and immediately closed, allowing only 0.2 cc. of fluid to enter. Furthermore, holding the burette open, until 5 cc. of fluid had entered the ventral aorta, did not increase or continue the initial inhibition during the period of flow. It follows that the effective stimulus is the initial increase of pressure *per se*, and not flow of fluid through the gill vessels. This was substantiated by blocking the flow through the gill capillaries so that the application of pressure alone served to stimulate. Attempts to do this by tying the efferent branchial supply from the hemibranch and first holobranch were unsuccessful because of the numerous anastomoses between these vessels and the neighboring efferent system. An effective block on the afferent side was obtained by occluding the gill capillaries with cornstarch, injected in suspension in urea-saline solution, through the ventral aorta. This suspension was withdrawn from the larger afferent vessels with a syringe and the vessels were refilled with urea-saline, thus giving a vascular pocket on the afferent side against which pressure could be applied without passage of fluid (Fig. 1, C).

That the receptors for the cardio-inhibitory response to increase of pressure within the gill vessels are not limited to the first two branchial arteries was shown by an experiment in which the ventral aorta was ligated between its first and second branches, and pressure applied to the third, fourth and fifth branchial arteries through a cannula inserted through the conus arteriosus (Fig. 1, D).

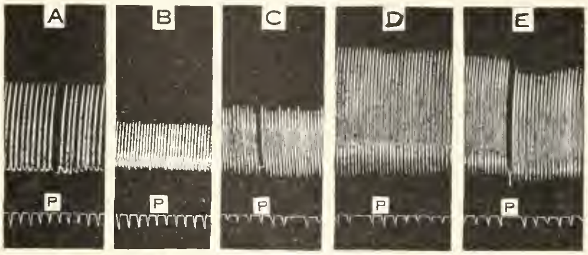

Fig. 3. Sudden increase of pressure within the first branches of the ventral aorta at *P*. Heart beat recorded by lever. A, before and B, after cutting the vagus supply to the heart on both sides. C. Vagus supply to heart cut on right side and increase of pressure in right gill vessels (crossed reflex). D, increase of pressure in right gill vessels, and E, in left gill vessels after cutting cranial nerves from fifth through tenth on right side (afferent supply from gills).

The cardiac inhibition following increase of pressure within the gill vessels is a reflex response inasmuch as it disappeared when the brain was destroyed by pithing. When the ramus posttrematicus of the fourth branchial division of the vagus (5th branchial nerve) and the ramus visceralis were cut on both sides the reflex was also abolished, thus showing that the efferent side of the reflex is in the vagus supply to the heart (Fig. 3, A, B). When these nerves were cut on the right side and the first right branch of the ventral aorta was clamped, thus leaving only the left gill vessels open to stimulation, the usual inhibition was obtained. When the clamp was shifted to the first left branch of the ventral aorta, the response was also obtained upon stimulating the right gill vessels (Fig. 3, C). Evidently the pathways for this reflex are both unilateral and crossed in the central nervous system. The threshold of pressure for the crossed reflex was about 10 cm. of urea-saline higher than that for the reflex obtained from stimulation of both branches.

The afferent innervation of the hemibranch and the first two gills was interrupted on both sides by cutting the first three branchial nerves (ninth and the first and second branchial divisions of the vagus). Following this no cardiac response to increase of pressure in both first branches of the ventral aorta was obtained. The integrity of the remaining cardio-inhibitory reflex mechanism was demonstrated by obtaining reflex inhibition upon pinching with forceps the skin between the fourth and fifth gill slits, the snout, the base of the pectoral fin, or upon cutting open the abdomen or scratching the wall of the pericardial chamber with a needle. The afferent side of the reflex following increase of pressure within the anterior gill vessels is thus located.

Inasmuch as cutting the first three branchial nerves involves opening the anterior cardinal sinus with considerable hemorrhage, the afferent nerve supply from the gills was interrupted on one side by transecting the cranial nerves from the fifth through the tenth close to the brain with a single incision through the skin. Following this, the usual cardio-inhibitory reflex was not obtained from the gill vessels on the operated side. Mechanical stimulation of the gill region on this side also failed to elicit a reflex, but both increase of internal vascular pressure and mechanical stimulation of the gills on the intact side evoked the cardio-inhibitory response (Fig. 3, D, E).

DISCUSSION

The blood pressure in the elasmobranch has been found to be of the low pressure type. Thus Schoenlein (1895) found in *Torpedo* a branchial systolic pressure of 16 to 18 mm. Hg and in *Scyllium* one of 30 to 33 mm. Hg; Hyde (1908) found the mean pressure in a branch of the aorta of the skate to be 20 mm. Hg; Lyon (1926) found in sand sharks an average branchial pressure of 32 mm. Hg and an average dorsal or systemic pressure of 23.3 mm. Hg. In *Squalus acanthias* we found the average pressure in the ventral aorta to be 28.2 mm. Hg and that in the dorsal aorta to be 15.4 mm. Hg. The average pulse pressure in the ventral aorta, as recorded by the mercury manometer, was 13.3 mm. Hg which is 47.2 per cent of the average systolic pressure. The ventricular beat as a factor in maintaining blood pressure is obviously important. This is in accordance with the fact that no vasomotor innervation has been demonstrated in these fishes. The heart reflexes, therefore, must be of importance as regulatory factors for the vascular needs of the body. The cardio-inhibitory reflex, as shown above, can be elicited by an increase in gill blood pressure well within physiological limits, and may temporarily decrease the diastolic blood pressure to a significant degree. In one case this decrease was 4 mm. Hg, which was 50 per cent of the diastolic pressure. Such a mechanism might come into play during an ejection reflex, when the sudden constriction of the branchial muscles forces water from the pharynx. The accompanying external pressure on the gill vessels would force blood from them in both directions. The blood pressure in the ventral aorta would rise, inasmuch as the blood would meet the valves in the conus arteriosus, evoking a sudden need for cardiac inhibition to prevent dangerous consequences, such as injury to the thin-walled afferent system. The increase in internal pressure would evoke the reflex, momentarily preventing additional pressure increase due to the ventricular action. As a matter of fact, our records show an increase of ventral aortic blood pressure well above threshold value followed by cardiac inhibition during spontaneous ejection reflexes (Fig. 2, D). This mechanism, therefore, being of physiological significance, may be compared with the carotid sinus mechanism in mammals. Cardiac inhibition during an ejection reflex may also be evoked by the mechanical stimulation of the gill region, inasmuch as the receptors for the heart reflexes have a wide distribution in the elasmobranch.

It is conceivable that in the course of evolution the widespread sensitive areas of the ancestral form, possibly typified by the elasmobranch, were concentrated or delimited until the condition seen in the mammal was reached. The carotid arteries of the mammal are derivatives of the primitive branchial system. The cardio-inhibitory reflex following increase of pressure within the gill vessels in elasmobranchs may exemplify, therefore, the evolutionary forerunner of the carotid sinus mechanism of mammals as it existed in whatever may have been the ancestral form. This is one of many instances in which it is apparent that physiological as well as morphological factors should be considered in evolutionary reasoning.

SUMMARY

1. Cardiac inhibition follows sudden increase of pressure within the gill blood vessels of *Squalus acanthias.* An average increase of 10.7 mm. Hg above the average systolic pressure existing in the dorsal aorta constitutes an adequate stimulus for the inhibitory response.

Respiratory reflexes were frequently seen associated with the cardio-inhibitory reflex induced by changes in blood pressure. Inasmuch as the carotid-sinus mechanism in mammals has recently been shown to be concerned in the regulation of respiration, further work on the peripheral control of respiration through receptors within the gill vessels of elasmobranchs is in progress.

2. The cardio-inhibitory response is a reflex, with afferent pathways located in the branchial nerves and efferent pathways in the vagus supply to the heart. The reflex is both unilateral and crossed.

3. The average ventral aortic systolic blood pressure in *Squalus acanthias* is found to be 28.2 mm. Hg, the average dorsal aortic systolic pressure 15.4 mm. Hg, and the average ventral aortic pulse pressure 13.3 mm. Hg. The inhibitory reflex to increased ventral aortic pressure modifies the diastolic blood pressure to a significant degree.

4. The adaptive nature of the reflex is pointed out and its phylogenetic significance is discussed.

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THE EFFECT OF ADRENALIN ON THE BLOOD PRESSURE OF THE ELASMOBRANCH, SQUALUS ACANTHIAS

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In elasmobranch fishes the distribution and relative preponderance of the components of the autonomic nervous system and the effects of adrenalin on the heart and digestive tract differ considerably from the same factors in mammals, as pointed out by Lutz (1930*a*, 1930*b*, 1931). The effect of adrenalin (1 : 50,000 to 1 : 25,000) on the heart was found to be inhibitory and was interpreted as the response of an unbalanced parasympathetic mechanism in an organ lacking a sympathetic accelerator innervation (Lutz, 1930*a*). The well-developed chromaphil system seen in elasmobranchs (Lutz and Wyman, 1927) leads one to believe that it must be of functional significance, and a possible inhibitory emergency theory has been suggested to account for its existence (Lutz, 1930*a*). The lack of accelerator nerves to the heart and the failure to demonstrate a vasomotor innervation to the blood vessels makes the abundance of chromaphil tissue in these fishes of especial interest to the physiologist. The well-known effect of adrenalin on the blood pressure of mammals, an effect which is relatively brief and readily duplicated by successive doses, appears to differ from its effect on the blood pressure of cold-blooded animals. Bieter and Scott (1929) reported a rise of blood pressure in the frog following a dose of 0.2 cc. of epinephrine hydrochloride, 1 : 10,000, which persisted for at least one and one-quarter hours after the injection. MacKay (1931) has found that adrenalin causes a rise of ventral aortic blood pressure in skates (*Raia*), which is of much longer duration than the pressor effect in mammals. Herein are reported the results of a preliminary investigation of the effects of adrenalin on the vascular system of the elasmobranch, *Squalus acanthias*. In view of the system of branchial capillaries located between the ventral and dorsal aortæ, simultaneous records of the blood pressures in both systems were considered essential.

MATERIAL AND METHOD

Specimens of the dogfish, *Squalus acanthias*, 730 to 1400 grams in weight, taken from Frenchman Bay, Maine, were used. The spinal cord was pithed posteriorly from the level of the sixth vertebra.

The fish was secured in a tank of sea water and the gills were perfused through the mouth as described in a previous paper (Lutz and Wyman, 1931). Simultaneous ventral and dorsal aortic blood pressures were recorded on a smoked drum by means of two mercury manometers and two cannulas, one inserted in one of the first branches of the ventral aorta thus leaving the second, third and fourth holobranchs on that side and all the gills on the other side for respiration, and the other inserted in the coeliac artery close to the dorsal aorta. The cannulas and manometer connections were filled with a physiological solution (urea-saline, Lutz, 1930*a*) containing a little heparin to prevent clotting. Adrenalin chloride (Parke, Davis and Co.), diluted in urea-saline solution, was injected into the portal vein. MacKay (1931) has shown that in the skate the effects of adrenalin administered in this way are the same as those obtained by injection into a large systemic vein. Control injections of urea-saline solution and adequate controls for manipulation during injections were carried out.

TABLE I

Effect of Initial Doses of Adrenalin

Exp. no.	Dose of adrenalin	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
		Syst.	Diast.	Pulse	Syst.	Diast.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
30	1 cc. 1 : 1000	71.5	40.0	150.0	75.0	—	−6
31	1 cc. 1 : 1000	29.1	30.8	27.8	53.8	72.8	+4
35	2 cc. 1 : 1000	70.0	46.1	114.1	91.0	80.0	0
36	2 cc. 1 : 1000	55.6	13.3	233.0	—	—	−30
27	2 cc. 1 : 10,000	39.2	38.5	40.0	111.8	133.3	0
29	2 cc. 1 : 10,000	60.0	33.3	100.0	66.5	—	−4
33	2 cc. 1 : 50,000	32.1	20.0	63.6	50.0	50.0	0
34	2 cc. 1 : 100,000	33.3	14.3	60.0	45.5	36.3	−4
37	2 cc. 1 : 500,000	33.3	42.8	23.1	91.6	111.0	+4

RESULTS

Intravenous injections of adrenalin, in doses of one or two cc. of 1 : 1000 to 1 : 500,000, produced rises of ventral and dorsal aortic blood pressure, both systolic and diastolic, persisting for at least 30 minutes (Table I; Fig. 1, A). No attempt was made to determine the maximum duration of the pressor effect, but MacKay (1931) has found it to be from one to two and one-half hours in the skate. There was also a marked increase of pulse pressure. The effect on the heart rate was not constant, but there was a general tendency

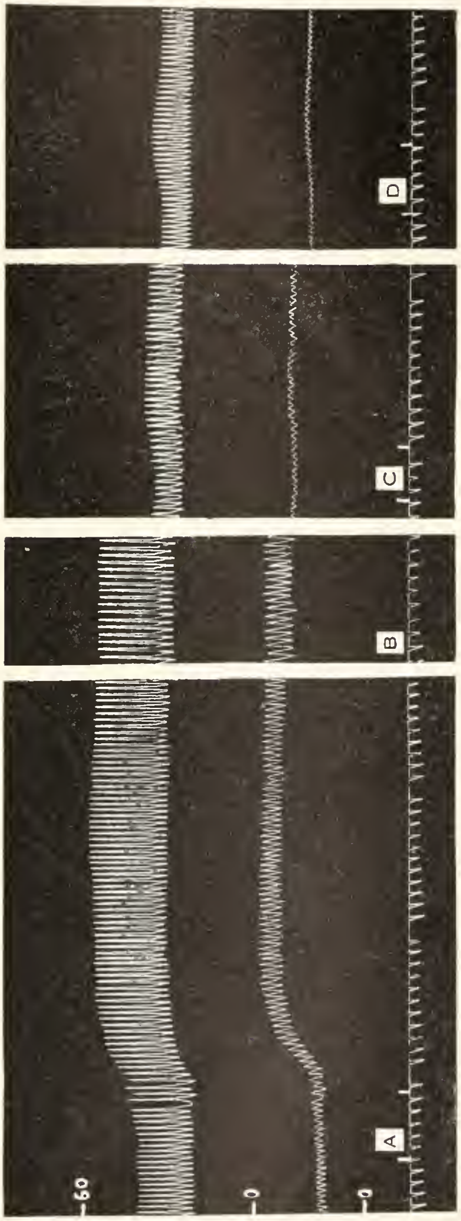

FIG. 1. The effect of adrenalin on the ventral (upper) and dorsal (lower) aortic blood pressures of *Squalus acanthias*. Cord pithed posteriorly from the level of the sixth vertebra. Time record shows five-second intervals. Figures at left show pressure levels in mm. Hg. The zero level (upper) for the ventral aorta is at 40 mm. Hg for the dorsal aorta. A. Exp. 27. Intravenous injection of 2 cc. of adrenalin, 1 : 10,000. B. Blood pressures 32 minutes later. C. Injection of 2 cc. of adrenalin, 1 : 10,000, 41 minutes after the initial injection (A). D. Exp. 33. Control injection of 2 cc. of urea-saline solution.

toward a decrease, especially following the stronger doses (1 : 1000, 1 : 10,000).

Subsequent doses of adrenalin following doses stronger than 1 : 500,000 produced small temporary increases of systolic pressure with little or no effect on the diastolic pressure, and consistent small increases of pulse pressure (Table II; Fig. 1, C). The rise of systolic pressure was, in most cases, actually due to the increase of pulse pressure. The effect of subsequent doses on the heart rate, however, was similar to that of initial doses.

TABLE II

Effect of Subsequent Doses of Adrenalin
(For initial doses see Table I)

Exp. no.	Dose of adrenalin	Time after first dose	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
			Syst.	Diast.	Pulse	Syst.	Diast.	
		min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
27	1 cc. 1 : 1000..........	33	1.8	11.1	4.3	17.1	16.7	−2
	2 cc. 1 : 10,000........	41	2.7	0	8.3	3.8	0	0
	2 cc. 1 : 10,000........	51	11.8	4.8	23.0	4.5	0	−2
29	2 cc. 1 : 10,000........	7.5	2.6	0	5.9	4.4	—	−6
33	2 cc. 1 : 50,000........	10	5.7	16.1	−5.9	15.0	23.5	0
34	2 cc. 1 : 100,000.......	3.5	3.1	0	6.2	6.2	6.7	+2
	2 cc. 1 : 100,000.......	8.5	3.1	0	6.2	6.7	0	−4
	2 cc. 1 : 1000..........	11.5	8.8	6.2	11.1	12.5	13.3	−4
37	2 cc. 1 : 500,000.......	9.5	20.0	11.1	33.3	33.3	12.5	0
	2 cc. 1 : 500,000.......	22.5	16.1	12.5	20.0	37.4	33.3	0
	2 cc. 1 : 1000..........	28.5	12.5	12.5	12.5	41.1	23.1	−6

Control injections of 2 cc. of urea-saline solution gave results which were essentially similar to those following subsequent doses of adrenalin with respect to blood pressure and pulse pressure. The heart rate, however, was unaltered (Table III; Fig. 1, D). It is probable, therefore, that such subsequent doses of adrenalin have little or no effect on the blood pressure.

After a dose of two cc. of adrenalin, 1 : 500,000, which gave a less prolonged pressor effect than stronger initial doses, subsequent doses of the same size were effective, producing significant increases of blood pressures and of pulse pressure which again were of shorter duration than the pressor effects of stronger doses (Table II, Exp. 37). This suggests that the ineffectiveness of adrenalin given during the prolonged pressor effect following stronger doses is due to the existence of the maximum pressor action of which the vessels are capable. Such an explanation obviates the necessity of assuming that a pharmacological tolerance to adrenalin has been acquired.

Excluding cardiac effects, a change in ventral aortic pressure following the administration of adrenalin might be due to alterations in either the gill vessels or in the systemic vessels. The increase of blood pressure following initial doses of adrenalin was often associated with slowing or with no change in heart rate. The percentage increase of diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta (Table I). These facts suggest a vasoconstrictor action of adrenalin, peripheral to the gill capillaries. Neither recording the venous outflow from the excised spiral valve (the blood vessels of which were perfused with adrenalin solutions) nor microscopic observation of the minute vessels of the tail, during the injection or direct application of adrenalin, gave evidence of vasoconstriction. Further work is necessary to locate the region of action of adrenalin in producing the pressor effect in *Squalus acanthias*.

TABLE III

Effect of Control Injections of Urea-Saline Solution

Exp. no.	Dose	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
		Syst.	Diast.	Pulse	Syst.	Diast.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
31	2 cc.	−3.1	0	−5.2	0	0	0
33	2 cc.	16.7	5.0	40.0	17.6	12.5	0
33	2 cc.	9.6	5.0	18.2	10.5	5.5	0
34	2 cc.	4.2	−12.5	37.5	9.1	—	0
37	2 cc.	11.5	0	23.1	9.1	0	0
37	2 cc.	3.1	0	6.2	5.8	0	0

There is considerable doubt concerning the existence of a sympathetic vasoconstrictor innervation in elasmobranchs. The long sustained pressor action of small amounts of adrenalin and the accelerator and augmentor action of minute doses on the heart of the skate (Huntsman, 1931) suggest that a possible function of the well-developed chromaffin system is to take the place of sympathetic nervous factors in vascular regulation. However, the discovery by MacKay (1931) of pressor changes in the ventral aorta following sensory stimulation in spinal or anesthetized skates, which were too brief to be due to reflex discharge of adrenin, invites further search for vasoconstrictor nerves.

SUMMARY

1. Intravenous injection of adrenalin, in doses as low as 2 cc. of 1 : 500,000, produced long-sustained pressor effects in *Squalus acanthias*, together with marked increase of pulse pressure and a tendency toward decrease of heart rate.

2. Subsequent doses of adrenalin following doses stronger than 1 : 500,000 were ineffective. This is interpreted as being due to already existing maximum pressor action. Doses subsequent to a dose of 2 cc. of 1 : 500,000 were effective.

3. The pressor effect is interpreted as being due to extra-cardiac factors, peripheral to the gill capillaries, but the region of action of the adrenalin was not located.

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PROPERTIES OF THE BLOOD OF THE SKATE (RAIA OSCILLATA)

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The system developed in the skate for transport of oxygen and carbon dioxide is similar in some respects to that of man. Oxygen combines reversibly with hemoglobin and probably passes in and out of the blood by diffusion. Carbon dioxide, transported as bicarbonate, is prevented from greatly changing the blood reaction by the buffer function of proteins.

If one seeks a more detailed description of the skate's respiratory system, it is found to differ from that of man in many respects. Thus a labile body temperature introduces a degree of freedom in the skate's blood which is absent in the blood of normal man. Other differences depend upon the physical state of the environment. In the one case the blood is separated by a membrane from a moving liquid. From this liquid, oxygen in solution diffuses into the blood stream; into it, carbon dioxide passes directly. In the other case the lung, acting as a buffer between the blood and a variable external environment, maintains air nearly constant in temperature and composition in the ultimate areas where gas exchange takes place. Carbon dioxide passes from the blood, not into a virtual vacuum, as from the gills of the skate, but into a gas phase where the partial pressure of carbon dioxide fluctuates within narrow limits about a mean value of 40 mm. So much can be said by induction; it remains to be seen how well the properties of the skate's blood are adapted to the requirements.

The experimental methods used in this investigation have been described by Dill and Edwards (1931) and need not be discussed here. Two difficulties arose, both related to the character of the erythrocytes. These are very resistant to rupture and in determining oxygen content low values may be obtained because of incomplete hemolysis. When the quantity of saponin in the ferricyanide reagent was tripled, hemolysis was complete and oxygen could be determined satisfactorily. The other difficulty was a consequence of the high metabolic rate of these nucleated cells. After blood is equilibrated and sealed in sampling tubes a rapid decrease in its oxygen content occurs. Usually oxygen was determined immediately but occasionally delay was unavoidable. In order to correct for changes in oxygen content, the rate of oxygen consumption was determined at several temperatures.

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Since these observations on metabolic rate of the blood may be of some interest in themselves they have been tabulated in Table I. In this experiment the blood had been saturated at a temperature of 10.5° with an oxygen partial pressure of 200 millimeters. Assuming the same solubility as in human blood of the same water content, dissolved oxygen amounted to 0.82 volume per cent and combined oxygen, 4.74 volumes per cent. With this information and the data of Table I and of Fig. 1 on the metabolic rates at 0, 20 and 40°, one can calculate the oxygen consumption of any specimen of blood within a wide range of conditions. It appears from Fig. 2 that the metabolic rate is not a linear function of the reciprocal of the absolute temperature. The observations are too few in number, however, to define this curve precisely.

TABLE I

Oxygen Consumption by Skate's Blood

Initial oxygen content = 5.56 volumes per cent

Time *min.*	Oxygen Content *vol. per cent*		
	0° C.	20° C.	40° C.
21			4.70
96			1.88
115			1.64
140		3.70	
375		1.37	
1000	4.29		

Observations have been made on the oxygen dissociation curves of three specimens of blood (two of which were composited from several skates) at four temperatures. A preliminary experiment was carried out in the usual way with variable oxygen pressures and with carbon dioxide partial pressures ranging from 10 to 100 millimeters. Since the blood has about one-half the buffer value of human blood it was supposed that this range of carbon dioxide pressures would have a greater effect on the position of the oxygen curves than in human blood. On the contrary, the effect was too small to be evaluated. A second experiment (Blood B; temperature 10.4°; Table II) was carried out with a greater range in carbon dioxide pressures,—from 0.5 to 140 millimeters. With this extreme range there appeared to be a small decrease in affinity for oxygen with increasing acidity.

TABLE II

Equilibrium Values for Oxygen Absorption

	pCO_2	pO_2	Total O_2	HbO_2
	mm. Hg	*mm. Hg*	*vol. per cent*	*per cent of capacity*
Blood A. HbO_2 capacity 5.68 vol. per cent	0.8	12.5	0.22	4
Temperature 37.5°	1.0	48.2	1.54	25
	0.8	128	3.97	64
	1.3	248	5.29	82
Temperature 25°	0.8	14.6	0.41	6
	0.7	31.3	1.79	30
	0.7	84.6	4.53	75
	1.0	100	4.55	75
Temperature 0.2°	0.5	2.4	0.60	10
	0.6	10.8	2.80	48
	0.5	13.8	3.65	62
	0.7	24.3	4.94	85
	0.7	48.6	5.68	96
	1.5	150	6.43	100
Blood B. HbO_2 capacity 5.54 vol. per cent	0.7	3.6	0.34	6
Temperature 10.4°	0.7	5.3	0.32	6
	0.5	8.1	1.00	18
	1.5	16	2.20	39
	0.5	26.5	3.62	64
	0.6	40.1	4.56	79
	0.8	58.8	5.15	89
	1.1	76.8	5.54	95
	2.3	190	6.30	100
	10.7	25.8	2.68	47
	140	23.2	2.29	40
	141	33.0	3.07	53
	137	40.7	3.41	59
Blood C. HbO_2 capacity 4.18 vol. per cent	2.2	108	2.60	56
Temperature 37.5°	2.7	254	4.13	84
Temperature 25°	0.6	23.2	1.24	28
	0.5	48.9	2.50	56
	0.5	83.1	3.19	70
	0.6	108	4.04	89
	1.0	165	4.49	95
	1.6	212	4.75	98
Temperature 10.4°	0.9	18.8	2.49	58
	0.5	22.3	2.42	56
	0.6	38.9	3.14	71
	2.2	167	4.95	100

This effect is represented quantitatively in Fig. 3. Ordinarily it is convenient to express the relation between position of the oxygen dissociation curve and pH by such an equation as

$$\log (X_{50}) = a(\text{pH}) + b,$$

where X_{50} represents the partial pressure of oxygen when the blood is 50 per cent saturated with oxygen, and the term a, the slope, is a measure of the rate of change of affinity of the blood for oxygen with rate of change in pH. This equation cannot be applied to our data on skate's blood since we do not have direct determinations of pH nor knowledge of the value of pK′ for serum or for cells. For our purposes, however, it is enough to use calculated values for log $[(\text{BHCO}_3)_b/(\text{H}_2\text{CO}_3)_b]$. This quantity differs from pH by a

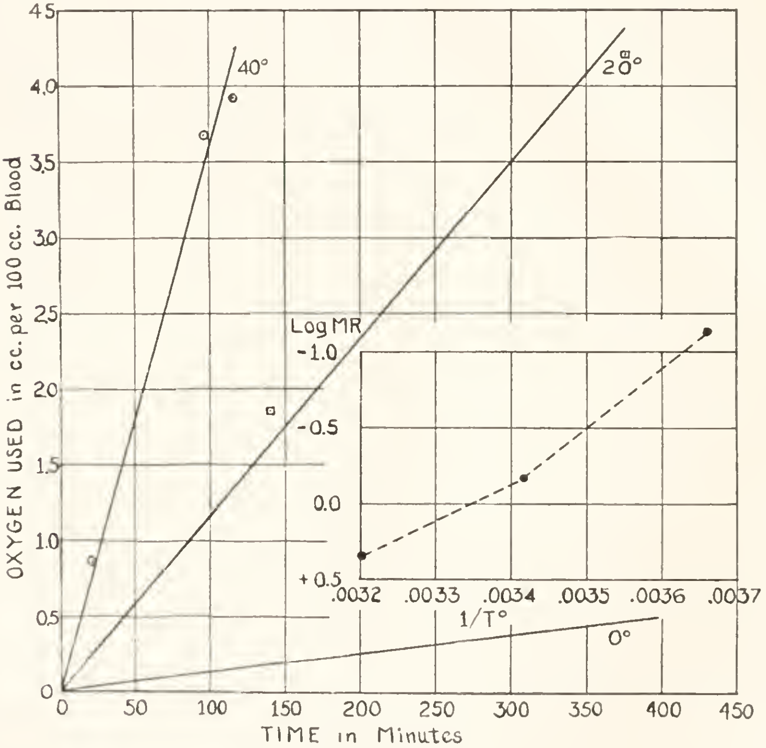

FIG. 1. Rate of oxygen consumption of normal skate's blood at temperatures of 0, 20, and 40°.

FIG. 2. (Inset.) Logarithm of the metabolic rate of skate's blood (oxygen used in cc. per hr. per 100 cc. blood) as a function of the reciprocal of the absolute temperature.

constant (or nearly constant) amount and hence the slope *a* will be essentially the same as though pH values were used. The curves for blood of the crocodile and of man shown in this figure are from Dill and Edwards (1931).

The curves for blood of the crocodile and for man are much steeper than for that of skate's blood. The contrast is greatest in the acid range; in fact within the range which corresponds to pCO_2 values from 10 to 140 mm. there is almost no change in position with change in reaction. However, the skate does not normally function within these limits but, as will be shown below, at a carbon dioxide pressure of one or two millimeters. In this range there is a distinct acid effect on oxygen affinity; here the slope *a* is roughly one-half as great as in man and one-fourth as great as in the crocodile. The blood of the skate is like that of *Urechis*, studied by Redfield and Florkin (1931), in the acid range but quite different when the reaction is more alkaline. They have found that the affinity of *Urechis* blood for oxygen is unaffected by change in hydrogen ion concentration when pCO_2 is varied from 0.5 to 92 millimeters. It would be interesting to speculate on the possible significance of these relationships in connection with structure of the hemoglobin molecule. However, we must remember

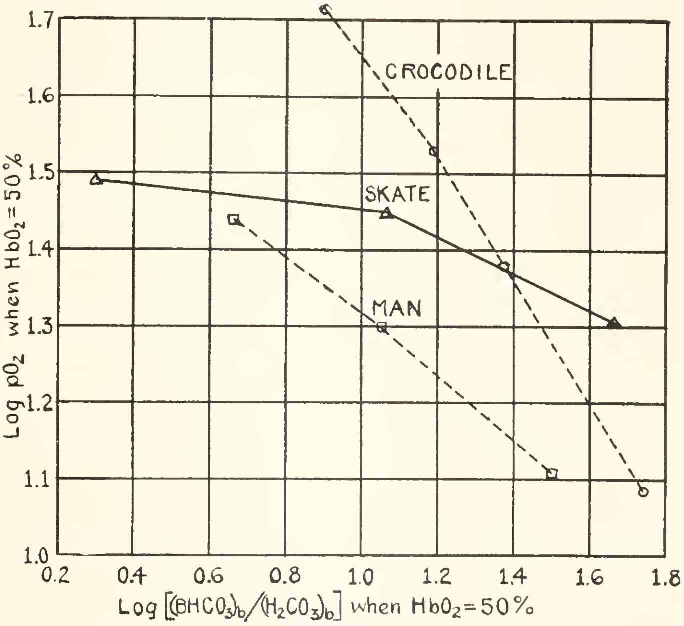

FIG. 3. Position of the oxygen dissociation curves (log pO_2 when HbO_2 = 50 per cent) in relation to log $(BHCO_3)/(H_2CO_3)$.

that the environment of hemoglobin is very different in these cases. It will be recalled that an abnormal value for a similar relationship was found in man in diabetic coma (Dill and others, 1929). Further discussion of this question had better wait, therefore, until it is possible to prepare these hemoglobins in the pure state and study them under strictly comparable conditions.

The data given in Table II have been used to construct the oxygen dissociation curves of Fig. 4. These have been drawn as members of the same family of curves, and aside from a few bad results the fit accords with this assumption. The effect of temperature on the affinity of blood for oxygen has been shown graphically for human blood by Brown and Hill (1923). Use has been made of their data and that of Fig. 4 in Fig. 5. This comparison indicates that *q*, the heat of reaction of 1 gram molecule of hemoglobin with *n* gram molecules of oxygen, is the same for the blood of the skate and of man. The significance of *n* remains somewhat obscure, but it is useful in characterizing the slope of the oxygen dissociation curve when $\log pO_2 = (f) \log Hb/HbO_2$. It has the value of 2.2 in man and in skate's blood it is slightly smaller, *viz.*, 2.0. If we accept the definition of *n* given by Brown and Hill, it follows that the value for *Q*, the heat evolved when 1 gram molecule of oxygen combines at constant volume with hemoglobin, is about the same as in human blood.

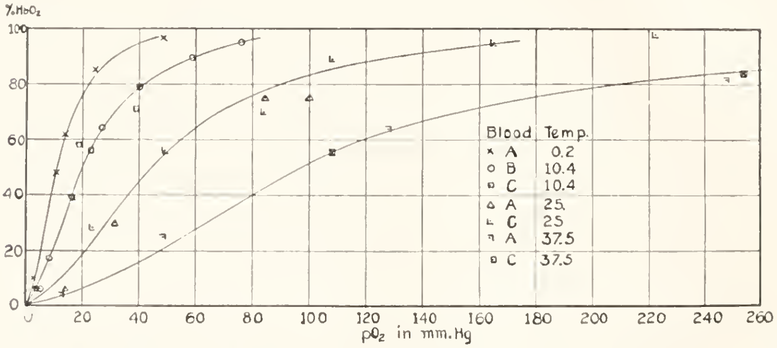

FIG. 4. Oxygen dissociation curves of skate's blood at temperatures of 0.2 10.4, 25 and 37.5° C. when $pCO_2 = 1 \pm 0.5$ mm.

One must have a description of the carbonic acid dissociation curve of blood in order to understand the conditions under which carbon dioxide is excreted through the gills. It is known from the work of Collip (1920), confirmed by others (Kokubo, 1927 and Smith, 1929), that the carbonic acid content of selachian blood is no more than 10 to 12 volumes per cent. Accordingly the carbonic acid dissociation curves have been studied over a low range of partial pressures. The curves for oxygenated blood are shown for temperatures of 10.4° (body temperature[2]) 25° and 37.5° in Fig. 6. The experimental procedure was simplified by the fact that, as in human blood, the relation of log (pCO_2) to log (Total CO_2) is linear or nearly so. Hence it was only necessary to determine a few points and fit the best straight line. The smoothed values were then transformed to the more familiar system of coördinates used in Fig. 6.

The alkaline reserve of blood, as suggested by Van Slyke and Cullen (1917), is most accurately defined by the bicarbonate content of arterial blood. When this is impractical, their method of equilibrating plasma of venous blood with alveolar air gives approximately correct values for human blood. The application of this method to fish blood, as by Collip (1920) and Kokubo (1927) does not define the alkaline reserve of fish blood but merely the carbon dioxide content at a partial pressure of carbon dioxide which is possibly twenty times greater than that of blood *in vivo.* It is probable that the values given by Collip for carbon dioxide content of blood equilibrated with atmospheric air measure the alkaline reserve more accurately than when alveolar air was used.

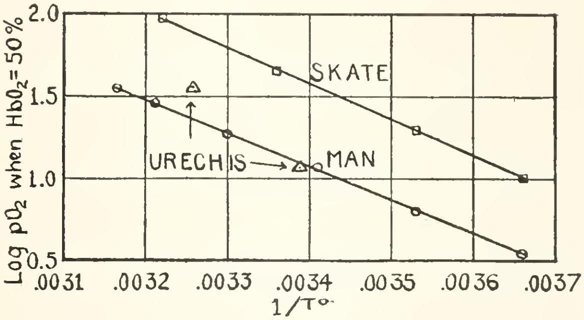

Fig. 5. Position of oxygen dissociation curves (log pO_2 when $HbO_2 = 50$ per cent) as a function of the reciprocal of the absolute temperature. The data for man are from Brown and Hill (1922–3) and for *Urechis,* from Redfield and Florkin (1931). In each case the oxygen is expressed in terms of partial pressures at the actual temperatures involved. The partial pressures of carbon dioxide were approximately 40 mm. for man, 1 for the skate and 12 for *Urechis.*

Having attained a description of oxygenated blood there remained to determine the effect of oxygenation on the position of these curves. There are several *a priori* reasons for supposing that this effect is too small to be measurable in skate's blood. These are: (*a*) the hemoglobin is one-fourth its concentration in normal human blood and the effect of oxygenation on the carbonic acid dissociation curves of blood will be reduced accordingly; (*b*) the alkaline reserve is lower and the distance between the curves will on this account be low, as may be seen from the curves for human blood in diabetic coma (Dill and others, 1929); (*c*) the effect of acid on the oxygen dissociation curves is much less in skate's blood than in human blood (see Fig. 3); accordingly

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[2] Body temperature was observed by rectum. It was maintained approximately constant by circulating sea water. Blood drawn at 10.4° and equilibrated at 25° and at 37.5° does not necessarily reflect the properties of blood drawn from animals acclimated to these higher temperatures. Possibly the blood would be altered in respect to available base and in other respects by change in body temperature.

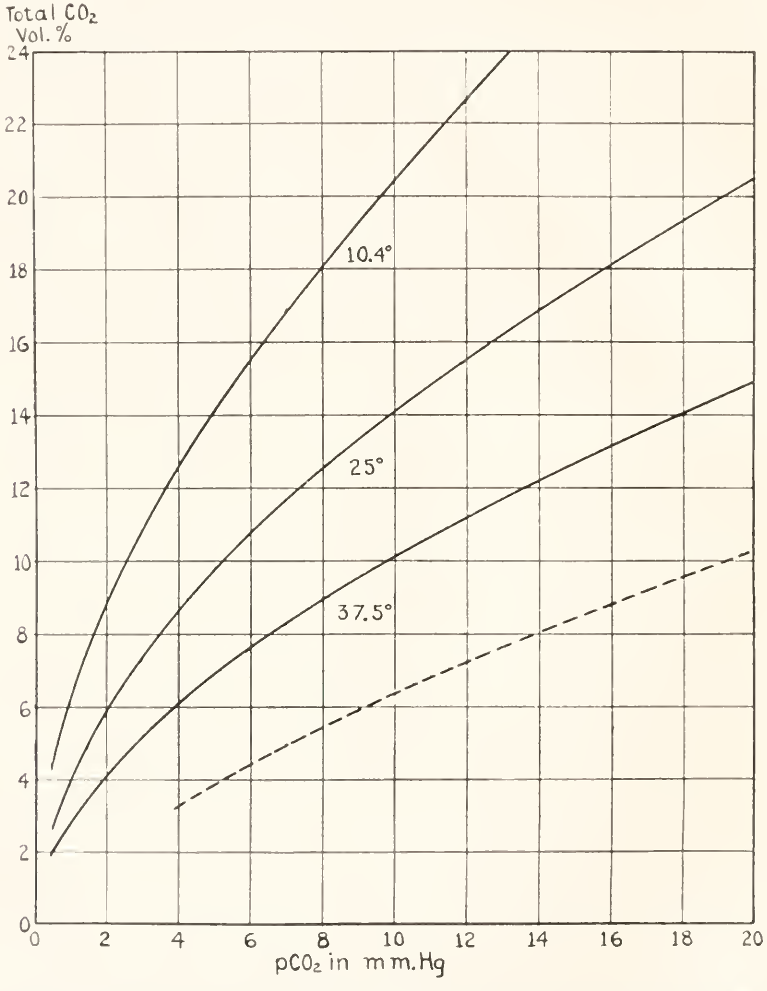

FIG. 6. Carbonic acid dissociation curves of skate's blood, body temperature 10.4°; equilibration temperatures, 10.4, 25 and 37.5°. The broken line corresponds to human blood at 37.5° in terminal chronic nephritis.

the effect of oxygen on the base bound by hemoglobin should be correspondingly less. For all these reasons taken together it would appear that oxygenation should have little effect on the carbonic acid dissociation curves and in fact several experiments, including one on concentrated blood, revealed no significant difference between the curves of oxygenated and of reduced blood.

It appears, then, that in the skate carbon dioxide is transported principally by virtue of buffering properties of blood proteins and we shall now direct our attention to that subject. It will be convenient to consider first the buffer value of separated plasma. The results of experiments in which the carbonic acid dissociation curves were used to calculate buffer value of plasma specimens are shown graphically in Fig. 7. Van Slyke's measure of buffer value is the rate of change of base bound with rate of change in pH:

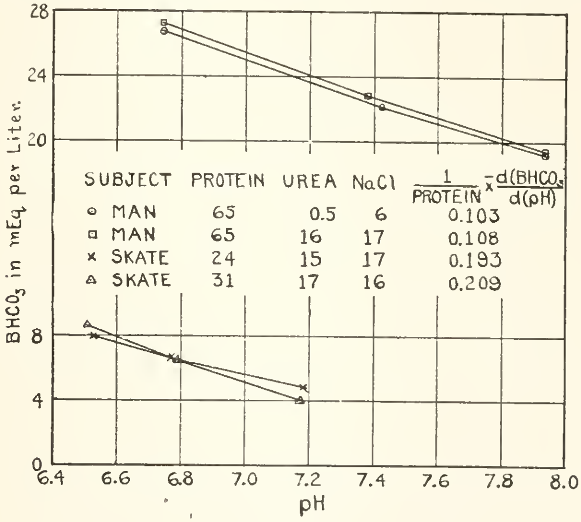

FIG. 7. Buffer value of skate's plasma and human plasma compared. Enough salt and urea was added to one specimen of human plasma to simulate skate's plasma in respect to those constituents. Concentrations of protein, urea and salt are in grams per liter.

$$\beta = \frac{dB}{d(\text{pH})}.$$

These curves define the buffering capacity of plasma, but since this is due principally to protein, it has been divided by the protein concentration in each case. The calculations inserted in Fig. 7 indicate an unusually high value for the buffer value of plasma protein of the skate. To determine the extent to which this is due to the high concentrations of urea and of sodium chloride in skate's plasma, a human plasma was prepared with concentrations of these substances typical of the skate. The effect of this modification proved to be very small and it follows that the proteins of the skate's plasma have, per gram, about twice the intrinsic buffering value of human plasma proteins. It will be noted that the protein concentration in the skate's plasma is from one-third to one-half as great as in human plasma. In effect, then, the buffer value of plasma per unit volume is almost equal in the two species.

It is possible to compare the buffer value of skate's whole blood with that of human blood by reference to the chart of Henderson and associates (1930, Fig. 3). The observations given in Table III are

TABLE III

Buffer Value of Oxygenated Skate's Blood at 37.5°

Blood	HbO_2 Capacity	CO_2 Capacity at $pCO_2 = 40$ mm.	$\Delta CO_{2_{60-30}}$		
			Observed	Calculated from human blood *	Ratio
	mEq. per l.	*mEq. per l.*	*mEq. per l.*	*mEq. per l.*	
Normal	2.0	8.4	3.3	3.1	1.1
Concentrated	4.7	7.0	3.15	3.2	1.0

* These values are calculated from the empirical chart of Henderson and associates (1930, Fig. 3).

based on one specimen of normal blood from the skate and one concentrated specimen obtained by centrifuging normal blood and removing about one-half the plasma. The comparison indicates that skate's blood has about the same buffer value as human blood of the same oxygen-combining capacity. Since the ratio of cell proteins to oxygen-combining capacity is about one-half greater in the skate than in man, it appears that the buffer value of cell proteins per unit weight is much less in the skate than in man.

This information regarding the physicochemical properties of skate's blood constitutes a suitable basis for study of changes in the respiratory cycle. The additional observations required are the oxygen and carbon dioxide contents of arterial and venous blood. We succeeded in obtaining for this purpose blood from the dorsal aorta and from the conus arteriosus while sea water at body temperature (9 to 10° C.) was being circulated over the gills. By application of the data thus obtained to the carbonic acid and oxygen dissociation curves it is possible to calculate the partial pressure of carbon dioxide and of oxygen in the blood *in vivo*. This and other calculations are shown in Table IV where a comparison also is made between respiratory changes in the skate and in man in terminal chronic nephritis (Henderson and others, 1927), a state which approximates in many respects to that of the normal skate.

The data given in Table IV have several points of interest. The striking difference as compared with man is in the carbon dioxide partial pressure. Man in terminal nephritis has a greatly increased rate of pulmonary ventilation but the partial pressure of carbon dioxide cannot be kept below 15 mm.; the skate, on the contrary, keeps the carbon dioxide pressure below 2 mm. in arterial blood. The steep character of the carbonic acid dissociation curve in this

TABLE IV

Comparison of the Skate with Man in Terminal Nephritis

	Skate *	Man †
Body temperature, ° *C.*	10.4	37.5
Cell count, *million per mm.*^{3}	0.2	1.0
Red cell volume, *cc. per 100 cc.*	20.0	14.7
Oxygen capacity, *vol. per cent*	6.00	5.60
Free oxygen, arterial blood, *vol. per cent*	0.32	0.28
Combined oxygen, arterial blood, *vol. per cent*	5.58	5.32
Combined oxygen, arterial blood, *per cent of capacity*	93	95
Free oxygen, venous blood, *vol. per cent*	0.07	0.06
Combined oxygen, venous blood, *vol. per cent*	1.91	1.62
Combined oxygen, venous blood, *per cent of capacity*	32	29
CO_2 content, arterial blood, *vol. per cent*	7.70	8.61
CO_2 content, venous blood, *vol. per cent*	10.84	11.75
CO_2 transport, *vol. per cent*	3.14	3.14
O_2 transport, *vol. per cent*	3.92	3.92
pCO_2, arterial blood, *mm. Hg*	1.3	15
pCO_2, venous blood, *mm. Hg*	2.6	23
pO_2, arterial blood, *mm. Hg*	70	110
pO_2, venous blood, *mm. Hg*	14	27
pH_s, arterial	7.82 ‡	7.00
pH_s, venous	7.67 ‡	6.95
ΔpH_s	0.15	0.05

* While these data given for the skate are based on a specimen of arterial blood from one skate and of venous blood from another, observations on other individuals have verified the approximate accuracy of the figures given in the table.

† These values have been obtained directly or by calculation from the study of terminal nephritis by Henderson and associates (1928). The values for carbon dioxide and oxygen transport have been arbitrarily made equal to the observed values in the skate. Associated changes in dependent variables have been read from the alignment chart for blood in nephritis.

‡ These values for pH_s are calculated on the assumption that $pK'_s = 6.24$ under the experimental conditions. The absolute values for pH_s may be incorrect, but the value for ΔpH_s will not be affected.

range makes possible the transport of 3 volumes per cent of carbon dioxide with a change in its partial pressure of only 1.3 millimeters. Another point of interest is the large change in pH of skate's blood. This is no doubt related to the greater effect in human blood of oxygenation on base bound by hemoglobin.

These facts are of particular interest in connection with the equilibrium between blood and air in respect to oxygen and carbon dioxide. The oxygen dissociation curve of skate's blood at 10.4° is approximately the same as man's at 37.5° and the oxygen tension in sea water is approximately the same as in air. The fact that arterial blood of the skate has about the same percentage saturation with oxygen as that of man indicates that the adequacy of oxygen transfer is approximately the same in the two species.

It has been shown by Bock and Field (1924) that in man the carbonic acid pressure is about the same in alveolar air as in arterial blood, most of the differences in partial pressure being within ± 1 millimeter. It is now possible to say that the partial pressure of carbon dioxide in the arterial blood of the skate exceeds that in water passing over the gills by no more than 1 or 2 millimeters. Collip has suggested that it is possible that a steep pressure gradient exists "*between the dissolved carbon dioxide in the blood on the one side, and in the sea water on the other.*" Such may be true of some bony fishes but it is not true of the skate.[3] His argument is as follows:

"As the hydrogen ion concentration of sea water is in most instances lower than that obtaining in the blood of marine forms and as the bicarbonate content of the latter is much higher than that of the former it is evident that the amount of the dissolved carbon dioxide in the blood or body fluids of marine forms must be considerably greater than that occurring in sea water. The tension of carbon dioxide in the blood of marine forms must also be proportionately higher than that in sea water."

This argument is sound provided one assumes that sea water is in equilibrium with atmospheric air in respect to free carbon dioxide. It may be misleading, however, because of the use of the word "considerably." Let us make a specific comparison of skate's blood and sea water:

	$BHCO_3$, *vol. per cent*	pCO_2, *mm. Hg*
Sea water	5	0.2
Arterial blood of the skate	8	1.4

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[3] In advance of its publication, we have had the privilege of reading the paper by Root (*Biol. Bull.*, **61**: p. 427), on the respiratory function of the blood of marine fishes. His single observation on arterial blood of *Prionotus carolinus* shows substantially the same pressure gradient of carbon dioxide from blood in the gills to sea water as we have found in the skate.

Assuming the same pK′, the blood of the skate will be more acid by 0.64 pH units. It is true that the ratio of carbon dioxide pressures is 7 to 1 but pressure gradient depends not on the ratio but on the difference in pressure. This difference, 1.2 mm., is small,—of the same order as in man.

SUMMARY

In the acid range, carbon dioxide pressure has almost no effect on affinity of skate's whole blood for oxygen. In the physiological range the effect is appreciable but still only one-half as great as in man. No difference was discerned between the carbon dioxide dissociation curves of oxygenated and of reduced blood. This was partly due to the facts that the hemoglobin concentration is one-fourth as great as in man and that the carbonic acid-combining capacity (when $pCO_2 = 40$ mm.) is less than in man.

The effect of temperature on the oxygen dissociation curves is identical with that found by Brown and Hill (1923) for human blood but somewhat different from that found by Redfield and Florkin (1931) for *Urechis* blood.

The buffer value of plasma proteins is about twice as great, per unit weight, as that of human plasma proteins. Since the concentration of protein in skate's plasma is one-third to one-half as great as in human plasma, it follows that the buffer value of plasma of the two species is about the same. Buffer value of whole blood is nearly equal to that of human blood of the same oxygen-combining capacity.

Transfer of gases between the blood and the external medium takes place under conditions which are quite different from those in the lungs of man. Nevertheless arterial blood is about equally saturated with oxygen in the two species. The absolute values for carbon dioxide pressure in man and the skate are very different because the blood of the skate is exposed to a virtual vacuum in respect to carbon dioxide. The pressure head of carbon dioxide from blood to the external medium, however, is of the same order of magnitude, about 1 mm. in each species. The supposition that there is a steep pressure gradient in respect to carbon dioxide in such a marine species as the skate is incorrect.

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THE NATURE OF THE REDUCING SUBSTANCES IN THE BLOOD SERUM OF LIMULUS POLYPHEMUS AND IN THE SERUM, CEREBROSPINAL FLUID AND AQUEOUS HUMOR OF CERTAIN ELASMOBRANCHS

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From the data in the literature it seems to be generally agreed that a variable part of the material determined as blood sugar is not glucose. Different investigators have attempted to determine glucose alone by using different reagents that react only with glucose, or by removing interfering substances (West, Scharles and Peterson, 1929) (Somogyi and Kramer, 1928). Folin and Svedberg (1926), because of discrepancies in the residual reducing substances after fermentation as determined by the Folin and Wu (1920) and Folin (1926) methods, say that the fermentable sugar is not all glucose. Both Benedict (1928) and Somogyi and Kramer (1928) conclude that there is no measurable amount of fermentable sugar other than glucose present in human blood.

We are presenting in this paper evidence to show that the reducing substances in the blood of *Limulus polyphemus* and in the blood, cerebrospinal fluid and aqueous humor of certain elasmobranchs are fermentable by yeast, and not appreciably affected by hydrolysis. We have determined the total reducing substances, reported as mg. glucose per 100 cc., by the method of Folin and Wu (1920) in the serum of *Limulus polyphemus* in twelve instances; in the serum of elasmobranchs in seven instances, in their cerebrospinal fluid in five, and aqueous humor in two instances. Yeast fermentation was done by the method described by Benedict (1928) in all these cases. The yeast blank which was subtracted from the residual after fermentation ranged from 4 to 6 mg. per 100 cc. Hydrolysis was done according to the method of Folin and Berglund (1922) in the twelve specimens of *Limulus polyphemus*.

We have already reported data on the relative composition of sea water and the blood of *Limulus polyphemus* (Dailey, Fremont-Smith and Carroll, 1931). The data reported here were obtained from the same samples of blood. The blood was taken by inserting a needle into the body cavity at the attachment of the caudal spine. In the elasmobranchs we endeavored to obtain samples allowing as little asphyxia as possible. The aqueous humor was obtained by puncture of the anterior chamber of the eye. Cerebrospinal fluid was obtained by puncture through the anterior fontanelle while the gills were still immersed in running sea water.[1] To collect blood the fish was then removed from water, the abdominal wall rapidly slit, the viscera pushed aside, and blood aspirated from the inferior vena cava. In animal 7, Table II, blood was obtained also from the abdominal aorta. The blood was collected under oil, centrifuged, and a "protein-free filtrate" made at once.

TABLE I

Reducing Substances in the Serum of Limulus Polyphemus

Animal	Date	Total Reducing Substances *mg. glucose per 100 cc.*	Residual after Fermentation *mg. glucose per 100 cc.*	After Hydrolysis *mg. glucose per 100 cc.*
1	8/12/29	10	0	10
2	8/12/29	11	3	13
3	8/13/29	15	2	15
4	8/13/29	11	3	15
5	8/13/29	12	1	11
6	8/13/29	14	3	18
7	8/26/29	14	0	14
8	8/26/29	21	1	21
9	8/26/29	16	0	21
10	8/29/29	13	2	13
11	8/29/29	22	0	22
12	8/29/29	15	1	19

The data on *Limulus polyphemus* are presented in Table I; those on the elasmobranchs in Table II. In the sera of *Limulus polyphemus* the residual reducing substances after fermentation varied from 0 to 3 mg. per 100 cc. and averaged 1.5. In the fluids from elasmobranchs there was in one instance a residual of 5 mg. per 100 cc., but the average was only 2 mg. per 100 cubic centimeters. The greatest change in reducing substances of the blood of *Limulus polyphemus* with hydrolysis was 5 mg. glucose per 100 cc., while in eight of the twelve cases there was a change of 2 mg. per 100 cc. or less. These changes are too slight to be of significance. Considering the limits of error of the methods used it may be said that the total reducing substances in serum, cerebrospinal fluid or aqueous humor examined

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[1] There is some question as to whether this fluid, obtained from the perimeningeal spaces, is truly analogous to the mammalian cerebrospinal fluid. (Smith, H. W., 1929. *Jour. Biol. Chem.*, **81**: 407.)

TABLE II

Reducing Substances in the Serum, Cerebrospinal Fluid and Aqueous Humor of Elasmobranchs

Animal	Date	SERUM		CEREBROSPINAL FLUID		AQUEOUS HUMOR	
		Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*	Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*	Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*
1. Mustelis canis	8/5/29	190	2				
2. Mustelis canis	8/7/29	157	1	107	1	50	0
3. Raia ocellata	7/18/29			23	0		
4. Carcharias littoralis	8/12/29	70	4	52	4		
5. Carcharias littoralis	7/29/29	70	1				
6. Carcharias littoralis	8/2/29	71	2	64			
7. Carcharinus obscurus	7/29/29	36 (aorta 73)	2 (aorta 5)	60	4		
8. Carcharinus obscurus	7/29/29	138	1	93	0	35	1

are fermentable by yeast and not changed by mild hydrochloric acid hydrolysis.

The striking difference in the level of total reducing substances in the blood of *Limulus polyphemus* and of the elasmobranchs studied is interesting in relation to the relative activity of the two groups. This relationship was discussed by Gray and Hall (1930). From their investigation we would expect to find low blood sugars in the sluggish and inactive *Limulus* and higher sugar values in the more active elasmobranchs. Our findings are consistent with theirs, as seen in Tables I and II. It is well-known that asphyxiation produces an increase in the amount of sugar in the blood of fishes (Denis, 1922; Menten, 1927; and Scott, 1921), which may in our cases have accentuated the higher blood sugar level of the elasmobranchs.

The fact that a fermentable reducing substance, not affected by hydrolysis, and therefore probably glucose, is consistently found in the blood of *Limulus polyphemus* and these elasmobranchs is interesting because there is little information as to the concentration or nature of the reducing substances in the blood of lower animals, and because of the ancient lineage of both species. We found the level of glucose in the sera of *Limulus polyphemus* to be relatively constant, varying only from 10 to 22 mg. per 100 cc. in the twelve animals studied.

There are some data in the literature on the blood sugar of elasmobranchs. Denis (1922) found the sugar of *Mustelis canis* to vary from 80 to 181 mg. per 100 cc.; Gray and Hall (1930) found 65 and 87 mg. per 100 cc. in two dogfish. These figures are within the limits of our findings, but we do not know the relationship of the blood sugar level, which varied from 36 to 190 mg. per 100 cc., to the degree of asphyxiation in the animals studied.

In Table II are reported five instances of parallel determinations of reducing substances in serum and cerebrospinal fluid. In four of these cases total and fermentable reducing substances were distinctly higher in venous serum than in cerebrospinal fluid. In case 7 the venous blood was lower, but the arterial higher. These studies give us no indication as to the relationship between reducing substances in the serum and cerebrospinal fluid at equilibrium as asphyxial elevations of the blood sugar had undoubtedly occurred after the cerebrospinal fluid was removed.

CONCLUSION

The reducing substance found in the blood serum of *Limulus polyphemus*, and in the blood serum, cerebrospinal fluid and aqueous humor of elasmobranchs is fermentable by yeast and not appreciably affected by hydrochloric acid hydrolysis. It is, therefore, probably glucose.

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STUDIES ON CELL METABOLISM

I. The Oxygen Consumption of Nereis Eggs Before and After Fertilization

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The sudden and enormous increase in the oxygen consumption of *Arbacia* eggs soon after fertilization, which was observed by Warburg (1908) and Shearer (1922*a*) and confirmed by measurements of the heat production (Shearer, 1922*b*, Rogers and Cole, 1925), had led to the assumption that the fertilization phenomena require a great expenditure of energy. Shearer correlates this increase to cortical changes in the egg and to the formation of the fertilization membrane. Loeb attributed the increase to the fact that the unfertilized eggs of the sea urchin are usually in the resting stage. To prove the validity of his hypothesis, he measured in collaboration with Wasteneys (1912) the oxygen consumption of fertilized and unfertilized starfish eggs, which as a rule are immature when taken out of the ovary, but as soon as they are placed in sea water may become mature. No noticeable increase in the rate of oxidation of fertilized starfish eggs was observed by these investigators, since they postulated "those oxidations which lead to nuclear division were already going on in the eggs at the time the spermatozoa entered." As Lillie and Just (1924) point out, with the formation of the hyaline plasma layer, the egg of *Arbacia* may be said to be well started on its first cleavage cycle. This would mean that "the measured oxygen consumption belongs rather to the physiology of cell division than to the more specific events of fertilization." (Lillie and Just, 1924.)

It was therefore of particular interest to the physiology of fertilization, to measure the respiratory changes of eggs in which this process can be observed from its initial stages. The eggs of the annelid *Nereis* are particularly suited to this purpose. As the excellent studies of Wilson (1892) and F. R. Lillie (1912) have established, the ovum of *Nereis* eggs is inhibited at the end of its period of growth. The germinal vesicle undergoes none of the preparatory stages of maturation unless the egg be fertilized. The spermatozoön remains more or less quiescent within the egg during the completion of the maturation division and the internal events of fertilization are resumed after the formation of the second polar body.

EXPERIMENTAL

The experiments were performed in Woods Hole during the summers of 1929 and 1930. Those of 1929 were done with the collaboration of Dr. A. Tyler. All the experiments were performed with freshly-caught *Nereis*. Large flat-bottomed Warburg vessels with Barcroft micro-manometers were used for the measurement of the oxygen consumption. The temperature of the water bath was kept at 25° C. The eggs were placed in the main side of the vessels. In some cases the sperm were placed in the side arm and fertilization was performed by pouring the sperm into the main side of the vessel, after the oxygen consumption of the unfertilized eggs had been measured. In other experiments, the sperm were added directly by opening the vessel. The results obtained by both methods were identical. A total of forty-two experiments were performed.

TABLE I

Oxygen consumption of Nereis eggs, before and after fertilization. The oxygen consumption after fertilization was followed until the two-cell stage had been reached.

Oxygen consumption in cu. mm. per hour		Per cent division
Before fertilization	After fertilization	
44.5	45.2	70
37.0	35.1	65
51.3	48.2	69
53.1	52.4	60
48.1	49.2	63
10.8	9.1	68
6.8	6.5	70
14.6	15.0	70
13.5	13.9	69

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The oxygen consumption after fertilization was measured until the eggs had reached the two-cell stage, *i.e.*, for about 80 minutes. The maximum percentage of cell division observed was 70 per cent and the minimum 45 per cent. In Table I are given figures of some of these experiments, at hourly periods. Figure 1 shows the oxygen consumption at different intervals after fertilization. In most cases (about 80 per cent) there was an average increase of 25 per cent in the oxygen consumption during the first eighteen minutes after fertilization. According to Lillie's observations on the fertilization events of *Nereis*, this corresponds to the time when the fertilization cone has flattened out, but the spermatazoön is still external to the membrane. But if one compares the total oxygen consumption of fertilized eggs (from the moment fertilization takes place, until the two-cell stage has been reached) with the oxygen consumed by the same eggs when unfertilized in equal length of time, it is observed that there is no difference. The subsequent decrease in the oxygen consumption is possibly due to a diminution in the rate of diffusion of the CO_2 produced by the cells on account of the jell which surrounds them. The amount of jell secreted by the ovum increases from the time of fertilization.

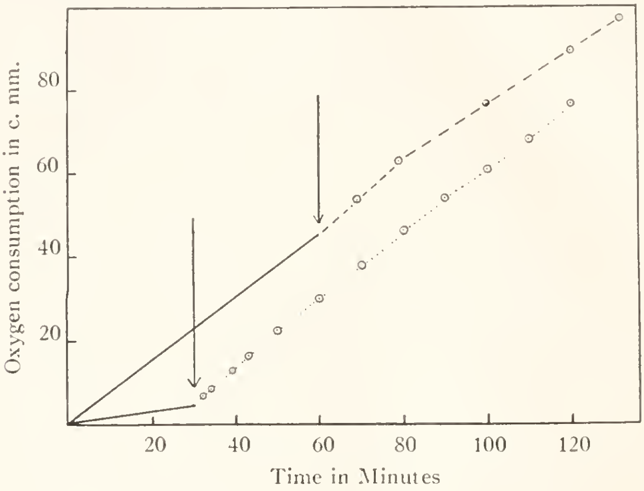

FIG. 1. The oxygen consumption of *Nereis* eggs before and after fertilization, as compared to the oxygen consumption of sea urchin eggs before and after fertilization. *Arbacia*, the lower line. *Nereis*, the upper line. Arrows mark the time of fertilization.

DISCUSSION

The striking increase in the oxygen consumption observed soon after *Arbacia* eggs have been fertilized has exercised such influence in the minds of most biologists, that one always finds it stated that the fertilization phenomenon is *in general* accompanied by an enormous increase in the oxidative processes of the fertilized cell. The case of fertilized starfish eggs, where such an increase is not observed, has not received corresponding attention. If Loeb's explanation for this lack of increased oxidation in fertilized starfish eggs is correct, one would expect in fertilized *Nereis* eggs about the same increase in the oxygen consumption as found in fertilized sea urchin eggs, since the initial process of fertilization starts here only when the spermatozöon enters the ovum. As has been shown in the experimental part, such is not the case. All one can say is that during the first eighteen minutes (which corresponds to the formation of the fertilization cone in the egg protoplasm while the spermatozoön is still found external to the substance of the entrance cone) there is a small increase of about 25 per cent, which is negligible compared to that found in fertilized sea urchin eggs where the oxygen consumption goes up to eight times that before fertilization. Fertilization in *Nereis* eggs proceeds without a decided increase in the cellular oxidative processes just as in starfish eggs. If expenditure of extra energy is needed for the performance of this activity, it is possibly supplied through a hydrolytic process, probably glycolysis. In the breakdown of glycogen to lactic acid an amount of energy is liberated, which according to Burk's calculations (1929) is from one and a half to two times greater than the heat of reaction. The great increase in the oxygen consumption of *Nereis* eggs after the addition of a reversible dye seems to support the view that these eggs possess a carbohydrate fermentation process, since the catalytic effect of dyes is mainly due to oxidation of carbohydrates which have already been rendered easily oxidizable by the action of enzymes (Barron, 1929).[1]

CONCLUSION

The oxygen consumption of *Nereis* eggs before and after fertilization to the two-cell stage has been measured.

1. The total oxygen consumption, from the initiation of fertilization to the two-cell stage, is no greater than the oxygen consumption for a similar period of time before fertilization.
2. When the oxygen consumption is measured at shorter intervals there is a small increase of about 25 per cent during the first eighteen minutes.
3. The present observations, together with those on the starfish eggs reported by Loeb, suggest that the enormous increase in oxygen consumption of fertilized sea urchin eggs may be considered an exception.

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[1] These experiments were performed with cresyl blue as catalyst. The increase in the oxygen consumption after dye addition is about 300 per cent.

THE EFFECT OF ANAËROBIOSIS ON THE EGGS AND SPERM OF SEA URCHIN, STARFISH AND NEREIS AND FERTILIZATION UNDER ANAËROBIC CONDITIONS

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The effect of anaërobiosis upon fertilization has been studied by various authors using the sea urchin as material for experimentation. All these studied have been confined to the effect of lack of oxygen after the sperm and the egg had been in contact, missing therefore the initial process of fertilization. Loeb and Lewis (1902) exposed the eggs of *Arbacia* soon after fertilization to lack of oxygen and stated that the process was inhibited. Mrs. Harvey (1927) subjected the eggs of the same species to a stream of hydrogen in a "modified Engleman's chamber" at different times after fertilization. In these experiments anaërobiosis was not reached according to the author until twenty minutes later. It is also possible that the sea water became hypertonic because of the small amount of fluid used in the chamber. More recently, Mrs. Harvey studied the effect of lack of oxygen on the eggs and sperm of sea urchin and on fertilization (Harvey, 1930).

In order to study the effect of anaërobiosis upon the fertilization phenomena, it is essential in the first place to select eggs and sperm of species which can resist the lack of oxygen without injury, at least during the time before they are brought in contact; in the second place, to use a technique where the absence of oxygen is controlled and where there is no danger of the sea water becoming hypertonic. It is the purpose of this paper to describe such a technique and to relate the effects of anaërobiosis upon the initial phase of fertilization of the following species: sea urchins, starfish and *Nereis*. These experiments were performed in Woods Hole during the summer of 1929.

METHOD FOR STUDYING THE EFFECT OF ANAËROBIOSIS ON FERTILIZATION

Three large specimen bottles (150 cc. capacity), tightly stoppered with rubber stoppers and connected to each other through glass tubing, were used for every experiment. Bottle A contained water in order to saturate the gas. The gas inlet was on bottle A. Bottle B contained the eggs suspended in 50 cc. of sea water. These eggs were previously received in large crystallizing dishes, washed twice with sea water and passed through cheesecloth. Bottle B contained a spoon where the sperm was kept when the fertilization was performed with sperm in anaërobiosis or a burette containing boiled sea water, when the fertilization was performed with fresh sperm. In the latter case there also was a platinum electrode and a saturated KCl bridge connected to a saturated calomel electrode. Bottle C contained a solution of safranin (approximate E′o − 0.290 v.) in Sorensen's phosphate buffer pH 8.0 and some platinum asbestos.

Test for Absence of Oxygen

It is essential in an experiment where the effect of anaërobiosis is followed, to be reasonably sure that no detectable amounts of oxygen are present in the vessels where the experiments are performed. In the early days reduced methylene blue had been used for such a purpose, but this is an unreliable test. The absence of luminescence in *Cypridina luciferin-luceferase* or in luminous bacteria has been advocated by Harvey (1926). When reversible systems showing a change of colour or light from the oxidized to the reduced state are used as tests for the absence of oxygen, it is necessary to have in mind that the rate of oxidation of the system is about proportional to the E′o of the system. Reduced indophenol will remain reduced for some minutes when the vessel containing the leuco dye is exposed to the air. Reduced methylene blue will oxidize more quickly but will remain reduced for a time in the presence of small oxygen concentrations. The more negative the E′o of the dye is, the more easily it will be oxidized by oxygen when in the reduced state (Barron, 1931). On these grounds the writer has used and recommends reduced safranin T as an excellent test for detecting the presence of minute traces of oxygen.

When fertilization was performed with fresh sperm the E. M. F. of the sea water obtained through a bright platinum electrode against a saturated calomel electrode was also used to detect the presence of oxygen. It is known that this electrode is extremely sensitive to oxygen.

Safranin was reduced with hydrogen and platinized asbestos. After complete reduction had been obtained, nitrogen (purified according to Michaelis and Flexner, 1928) was bubbled throughout the experiment. Experiments in which there was the slightest coloration of safranin were discarded.

The Effect of Anaërobiosis on the Eggs and Sperm of Sea Urchin and Fertilization under Anaërobic Conditions

Loeb (1915) stated that sea urchin eggs could be better preserved in absence of oxygen, and related experiments where eggs kept for twenty-four hours under hydrogen could be fertilized as well as fresh eggs. It is possible that in these experiments, the eggs were not exactly under anaërobic conditions but under low oxygen pressures. Loeb's experiment has been repeated, where purified nitrogen was passed through the vessel for a period of twenty-four hours. The eggs were then transferred to a flat-bottomed dish and fertilized with fresh sperm. There were many cytolyzed cells, and on fertilization the eggs gave only 15 to 20 per cent of cell division. Similar experiments performed with fertilized eggs soon after fertilization (the vessels were kept on ice while deaëration was completed) gave identical results, *i.e.*, although there was 100 per cent membrane formation, only 20 per cent of the eggs reached the two-cell stage. Many of these cells gave abnormal types of division. When the eggs are kept in anaërobic conditions for periods of one hour to six hours they can be normally fertilized with fresh sperm (Table I). From experiments on the effect of KCN on the life of sea urchin sperm, Cohn (1918), confirming similar observations of Drzewina and Bohn (1912), concludes that lack of oxygen preserves the life of these cells. When the experiments are performed in *true* anaërobiosis such is not the case. Sea urchin sperm are very sensitive to lack of oxygen. Concentrated sperm suspensions were kept in anaërobiosis for periods of one to five hours. It was observed that they could live, remain motile and fertilize fresh eggs only after one to two hours of anaërobiosis. Three hours later the sperm had lost their vitality and fertilized fresh eggs no more (Table I).

From what has been said about the extreme sensibility of sperm to lack of oxygen it is obvious that fertilization could not be possible when eggs and sperm were kept in anaërobic conditions. Such is the case. Fertilization was performed by introducing the spoon which contained the sperm suspension into the fluid containing the eggs while the vessel was shaken. The nitrogen flow was continued for one hour more. The vessel was opened, and immediately formol was added in sufficient quantity to make a 10 per cent solution. The eggs were examined microscopically and then fixed and stained for later observation.[1] Fertilization occurred in none of these experiments, as tested by the absence of membrane formation. The results, of these experiments are essentially similar to those found by Mrs. Harvey (1930) working at Woods Hole the same year in which these experiments were performed.

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[1] The author wishes to express his thanks to Dr. Fry, who generously had the microscopic sections performed in his laboratory, New York University.

Fertilization of anaërobic eggs with fresh sperm was performed by adding a drop of sperm to a burette containing boiled sea water. On account of their great density the sperm go to the bottom of the burette quite readily. The stopcock was opened and four drops of sperm added while the vessel was being shaken.

Anaërobiosis was tested with safranin and by measuring the E. M. F. with a bright platinum electrode. Safranin remained colorless during the whole experiment. The E. M. F. before fertilization was − 0.1805 volts (referred to the H_2 electrode). It remained constant for 15 minutes, after which it became a little more negative, − 0.1815. During the hour that nitrogen was passing, this E. M. F. rose to −0.184 and remained quite constant. The significance of such a negative potential and its bearing upon the reduction potential of the cell will be the subject of another paper.

TABLE I

The Effect of Anaërobiosis on the Eggs and Sperm of Starfish, Sea Urchin and Nereis, as Tested by Fertilizing Them with either Fresh Sperm or Eggs

Duration of anaërobiosis in hours	Starfish		Sea Urchin		Nereis	
	Eggs	Sperm	Eggs	Sperm	Eggs	Sperm
	Per cent fertilization					
1	90–100	90–100	90–100	90–100	90–100	90–100
2	90–100	90–100	90–100	2	90–100	90–100
3	65–50	50	90–100	None	90–100	90–100
4	None	None	90–100	None	90–100	90–100
5	None	None	90–100	None	90–100	90–100
6			90–100	None		
24			15–20			

Sperm and eggs remained under nitrogen for one hour. The vessels were opened and treated as indicated. A portion was examined immediately for membrane formation and the rest was kept for sectioning and staining. The results obtained were the following. There was membrane formation on the eggs in all experiments up to six hours anaërobiosis. In the stained sections the spermatozoön could be seen inside the egg cytoplasm at different distances from the membrane, but in no case was there any nuclear change in the ovum, which remained quiescent [2] (Table II).

The Effect of Anaërobiosis on the Eggs and Sperm of Starfish and Fertilization under Anaërobic Conditions

The metabolic changes accompanying the process of fertilization in these eggs are different from those in *Arbacia*. As Loeb and Wasteneys (1912) have shown, no increase in the oxygen consumption is observed. Loeb attributed this to the fact that the egg becomes mature before the entrance of the spermatozoön. While the eggs of the unfertilized sea urchin show very low metabolic activities, the eggs of the starfish possess a high metabolism. It could therefore be predicted that anaërobiosis would be harmful to the starfish egg, which requires the consumption of a great amount of energy for its normal activity. Starfish eggs kept in anaërobiosis are dead four hours after, as tested by the fertilization method.

TABLE II

Fertilization in Anaërobiosis

The eggs of starfish, sea urchin and *Nereis* have been kept in anaërobiosis during the duration of the experiment and been fertilized either with fresh sperm or sperm kept in anaërobiosis.

	Eggs	Sperm		
	Duration of anaërobiosis in hours	Duration of anaërobiosis in hours	Fresh	
Sea urchin.	1 to 5	1 to 5		No membrane formation.
	1 to 6		fresh	Membrane formation. Spermatozoön has penetrated inside the egg. Ovum quiescent. No nuclear changes.
Starfish.	1 to 5	1 to 5		No membrane formation.
	1		fresh	3 per cent membrane formation. No nuclear changes.
	2 to 5		fresh	No membrane formation.
Nereis.	1 to 3	1 to 3		Fertilization process proceeds for 15 minutes, after which it stops.
	1 to 5		fresh	Similar results.

The sperm of starfish are more resistant to the effect of lack of oxygen than the sperm of *Arbacia.* They can remain motile and fertilize fresh eggs after three hours of anaërobiosis. They are dead after four hours of anaërobiosis.

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[2] The author wishes to express his thanks to Dr. E. E. Just for examining these sections.

Since both the eggs and sperm of starfish are sensitive to lack of oxygen, it is obvious that they are unfit for the study of anaërobic fertilization. When fertilization was performed using anaërobic eggs and anaërobic sperm there was neither membrane formation nor penetration of the spermatozoön. When fertilization was performed with fresh sperm it was found that eggs kept anaërobically for one hour would give three per cent membrane formation, although, as in the case of sea urchins, no nuclear changes were observed in the stained sections. From two to five hours later there was no formation of membrane.

The Effect of Anaërobiosis on the Eggs and Sperm of Nereis and Fertilization under Anaërobic Conditions

The eggs of *Nereis* are the best material for the study of the initial process of fertilization, since the ovum of *Nereis* eggs is inhibited at the end of its period of growth. The germinal vesicle undergoes none of the preparatory stages of maturation unless the egg be fertilized. Fertilization of *Nereis* eggs is similar in its behaviour towards oxygen consumption to starfish, as there is no appreciable increase in the respiration of the egg after fertilization (Barron, 1931). The eggs of *Nereis* behave like those of the sea urchin in their resistance to lack of oxygen. They can be kept in anaërobiosis for periods varying from one to five hours. When fertilized with fresh sperm afterwards, normal fertilization can be observed in every case.

The sperm show the same resistance to lack of oxygen as the eggs. The sperm were kept in anaërobiosis from one to three hours and fifteen minutes. They were afterwards used to fertilize fresh eggs in normal sea water. In every case there was normal fertilization. With anaërobic sperm and anaërobic eggs, the fertilization experiments were followed from one to three hours. No sections were made and the microscopic observations were performed soon after formol fixation. The jelly, which starts to exude from the cytoplasm three to four minutes after fertilization and lasts for ten to twelve minutes, could be seen in the vessels. At the microscope the cells showed the sperm attached to the fertilization cone. Fertilization proceeded normally until the fifteen-minute period and stopped there. Similar results were observed when fresh sperm and anaërobic eggs were used. No nuclear changes were observed.

Discussion

The experiments performed by previous workers on the subject of anaërobic fertilization have dealt with only one kind of cell, namely, sea urchin cells. From the behaviour of sperm and eggs of three different kinds of animals, representing three different types of fertilization, a study which has been reported above, it is seen that in order to solve the problem it is essential to use the eggs and sperm of animals which are insensitive to anaërobiosis. Sea urchins cannot be used because the sperm are sensitive to lack of oxygen. Starfish cannot be employed because the eggs show identical sensitivity. On the other hand, the eggs and sperm of *Nereis* show a remarkable resistance to anaërobiosis, so that even four hours after anaërobiosis either sperm or eggs can fertilize normally when put in contact with fresh eggs or sperm in the presence of air. It has been shown that fertilization of *Nereis* eggs and sperm is initiated in anaërobic conditions and the process continues until the formation of the fertilization cone, a period corresponding to fifteen minutes. The process stops there. No nuclear changes are observed. The same phenomenon occurs in sea urchin eggs and starfish eggs when fertilization is performed with fresh sperm. The fertilization membrane is formed and the spermatozoön enters into the ovum, but no nuclear changes are observed. It can therefore be concluded that the initial process of fertilization, *i.e.*, penetration of the spermatozoön and formation of the fertilization membrane, is possible in strictly anaërobic conditions, provided the two cells which take part in such process have conserved their vitality. The process stops in this stage and no nuclear divisions take place. These findings seem to favor the hypothesis that if some energy is required for the initial process of fertilization it may be obtained through some hydrolytic process, possibly the breakdown of the carbohydrate molecule into lactic acid, which, as is well-known, does not require oxygen utilization.

The breakdown of glycogen into lactic acid produces an amount of energy which, according to Burk (1929), is from one and a half to two times greater than the heat of reaction. The fact that fertilization does not go beyond the entrance of the spermatozoön into the egg protoplasm and no nuclear changes are observed, may be due to inability of the lactic acid formed to be resynthesized into glycogen on account of absence of oxygen.

Conclusions

1. The eggs of sea urchin are insensitive to anaërobiosis for as long as five hours. After this time cytolysis of the eggs begins.

The eggs of starfish are more sensitive to lack of oxygen. Four hours after anaërobiosis the eggs of starfish are incapable of being fertilized. The eggs of *Nereis* can be kept under anaërobic conditions as long as five hours (maximum limit of our experiments) without any injury.

2. The sperm of sea urchin eggs are very sensitive to anaërobiosis. Two hours after anaërobiosis the fertilization power of the sperm is already diminished. Three hours after, the sperm has lost the power to fertilize. The sperm of starfish are less sensitive to anaërobiosis. They lose their fertilization power four hours after anaërobiosis. The sperm of *Nereis* can be kept as long as five hours in anaërobic conditions without any injury of their fertilization power.

3. Since, in order to reach conclusions regarding the possibility of anaërobic fertilization, it is essential that the two cells be insensitive to anaërobiosis, this condition is fulfilled only in the case of *Nereis*. Fertilization in *Nereis* eggs can be started with both eggs and sperm having been kept in anaërobiosis for five hours. The fertilization process is stopped with the formation of the fertilization cone, *i.e.*, which corresponds to fifteen minutes after normal aërobic fertilization has started. No nuclear changes are seen. Fertilization is started in sea urchin eggs when fresh sperm are used. The process stops with the formation of the fertilization membrane and the entrance of the spermatozoön into the egg protoplasm. No nuclear changes take place. In starfish eggs, anaërobic fertilization with fresh sperm is possible to a certain extent (membrane formation and entrance of spermatozoön).

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FAT METABOLISM OF THE CHICK EMBRYO UNDER STANDARD CONDITIONS OF ARTIFICIAL INCUBATION

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The bird's egg consists of about forty per cent of fat. The largest portion of the fat is found in the egg yolk. In addition to this, during incubation period some fat is synthesized from protein and some possibly from carbohydrate molecules. All this fat is utilized for nutrition and energy supply of the developing embryo and of the young bird after hatching.

Among the earliest workers with avian eggs Parke (1866) noticed that the amount of fat in the egg yolk diminishes during incubation. Then Eaves (1910), Sakuragi (1917), Idzumi (1924) and Murray (1926) showed experimentally that as the actual amount of fat decreases in a whole egg, it increases in an embryo. Needham (1925 and 1927) in his reviews of the literature pointed out that the fat in an egg is the most important energy-source of the developing embryo.

The above facts would suggest that the egg fat is of such chemical composition that it can be utilized according to the need of the embryo. The process of absorption of fat, particularly of yolk fat, also gives another aspect of the question. It looks very much as if there is a preferential absorption of the unsaturated acids by the embryo at certain stages of incubation (Eaves, 1910).

The present paper consists of a further experimental study of the fat metabolism of the chick embryo under standardized or "normal" conditions of artificial incubation, that is, changes of the amount and chemical nature of fat in the embryo and egg yolk as measured by the iodine value, saponification number and refractive index.

Methods and Materials

All eggs used were from a flock of White Leghorn hens (*Gallus domesticus*). The eggs were selected for uniformity of size and quality of eggshell (Romanoff, 1929).

The incubation was carried on in a special electric laboratory incubator (Romanoff, 1929*a*) under the conditions already described (Romanoff, 1930) with the temperature 38.0 ± 0.2° C., and the relative humidity 60.0 ± 1.0 per cent.

TABLE I

Iodine Value, Saponification Number and Refractive Index of Fat (ether extract) of Yolk of Fowl's Egg

Author	Percentage of Fat in Wet Weight	Iodine Value	Saponification Number	Refractive Index
	per cent			$(n)_D$
Parke (1866)	22.82	—	—	—
Amthor and Zink (1897)	—	75.4	189.4	1.4670
Kitt (1897)	19.00	71.1	190.2	—
Peninngton (1909)	32.39	63.6	182.2	1.4611
Serono and Pallazi (1911)	20.17	82.31	198.85	—
Thomson and Sorley (1924)	31.10	74.73	183.79	—
Romanoff (present rpt.)	30.71	73.89	190.69	1.4690

MARINE BIOLOGICA

At intervals of 24 hours all eggs were candled for dead embryos, and at least four eggs with normally developed embryos were removed for analysis.

Previous experience in our laboratory showed that at certain stages of embryonic development it is almost impossible to separate the yolk from albumen or allantoic fluid, and to determine it quantitatively with appreciable accuracy. Therefore the boiling of eggs,—to complete coagulation of yolk and albumen,—had been employed. This method proved to be very quick and satisfactory, not only in determining the total values of yolk and albumen, but also in some chemical analysis, such as fat (ether extract) determination.

TABLE II

Distribution of Fat (ether extract) in a Fresh and Hatched Egg

Parts of the Egg	Fat (ether extract)	
	Amount	Percentage
	grams	*per cent*
Fresh egg:		
Yolk	5.8275±1.0971	99.50
Albumen	0.0046±0.0013	0.08
Shell	0.0033±0.0025	0.06
Shell membranes	0.0213±0.0167	0.36
Total	5.8567	100.00
Hatched egg:		
Chick	1.8887±0.1224	48.57
Yolk sac	1.9315±0.0762	49.67
Shell with waste matter	0.0682±0.0126	1.76
Total	3.8884	100.00
Difference (combusted fat)	1.9683	33.61

TABLE III

Changes in the Fat (ether extract) Content of the Chick Embryo

Stage of Incubation	Weight of Embryo			Fat (ether extract) Content			
	Wet Weight	Dry Weight	Percentage of Dry Matter	Composed Samples	Average Fat Content	Percentage of Wet Weight	Percentage of Dry Weight
days	*grams*	*grams*	*per cent*	*embryos*	*grams*	*per cent*	*per cent*
8	1.350	0.099	7.33	18	0.0133	0.99	13.13
9	2.083	0.147	7.06	12	0.0233	1.12	15.85
10	2.700	0.238	8.81	6	0.0310	1.15	13.03
11	3.850	0.305	7.92	6	0.0539	1.40	17.67
12	5.499	0.513	9.33	5	0.1015	1.85	19.79
13	7.213	0.703	9.75	4	0.1610	2.22	22.90
14	9.670	1.159	11.99	2	0.2575	2.66	22.22
15	11.327	1.544	13.63	2	0.3475	3.07	22.51
16	15.360	2.582	16.81	2	0.5012	3.26	19.41
17	18.095	3.396	18.77	2	0.7997	4.42	23.55
18	22.310	4.220	19.92	2	0.9823	4.40	23.28
19	29.891	5.812	19.44	1	1.3024	4.36	22.41
20	31.298	5.999	19.17	1	1.4053	4.49	23.43

TABLE IV

Changes in the Fat (ether extract) Content of the Yolk Sac

Stage of Incubation	Average Egg Weight	Weight of Yolk Sac			Fat (ether extract) Content		
		Wet Weight	Dry Weight	Percentage of Dry Matter	Average Content	Percentage of Wet Weight	Percentage of Dry Weight
days	*grams*	*grams*	*grams*	*per cent*	*grams*	*per cent*	*per cent*
0	61.6	18.44	9.654	52.35	5.6633	30.71	58.66
1	62.0	19.19	10.110	52.68	6.4837	33.79	64.17
2	62.5	19.79	10.113	51.10	6.0309	30.47	59.54
3	62.5	20.10	9.546	47.49	6.0338	30.02	63.21
4	62.5	21.79	10.390	47.68	6.5621	30.12	63.16
5	61.3	20.22	8.781	43.43	5.3549	26.48	60.98
6	61.8	21.51	8.872	41.24	5.4276	25.23	61.18
7	62.3	21.81	9.354	42.88	5.8198	26.68	62.22
8	62.5	20.58	8.611	41.84	5.4874	26.66	63.73
9	60.3	19.84	8.726	43.98	5.6041	28.25	64.22
10	62.0	19.08	8.767	45.95	5.2271	27.39	59.62
11	61.3	18.80	8.382	44.59	5.3328	28.37	63.62
12	61.5	18.27	8.617	47.17	5.4158	29.64	62.85
13	62.0	18.31	9.097	49.68	5.4335	29.68	59.72
14	59.3	16.64	9.539	57.35	5.5897	33.59	58.59
15	62.5	16.34	9.676	59.25	5.4803	33.55	56.64
16	60.0	14.54	9.006	61.94	4.5241	31.11	50.23
17	61.0	14.02	9.132	65.14	4.4922	32.04	49.19
18	62.5	13.87	8.255	59.52	3.0893	22.27	37.42
19	59.5	12.03	6.986	58.57	2.4567	20.42	35.17
20	60.5	10.53	6.015	57.12	2.1434	20.35	35.64

In these experiments two eggs were boiled for 20 minutes, the coagulated yolk was weighed, then dried to constant weight in a Freas electric vacuum oven at 55° C. and vacuum at about 63.5 cm., ground and subjected for 48 hours to extraction of fat with anhydrous ether by a Soxhlet extraction apparatus.

From two or more unboiled eggs the embryos were separated for determination of dry matter and fat content by methods similar to those described above.

The extracted fat from both the embryo and the yolk sac was tested for saponification number (Koettstorfer, 1879), iodine value (Wijs, 1898),[1] and refractive index, by a Zeiss butter refractometer.

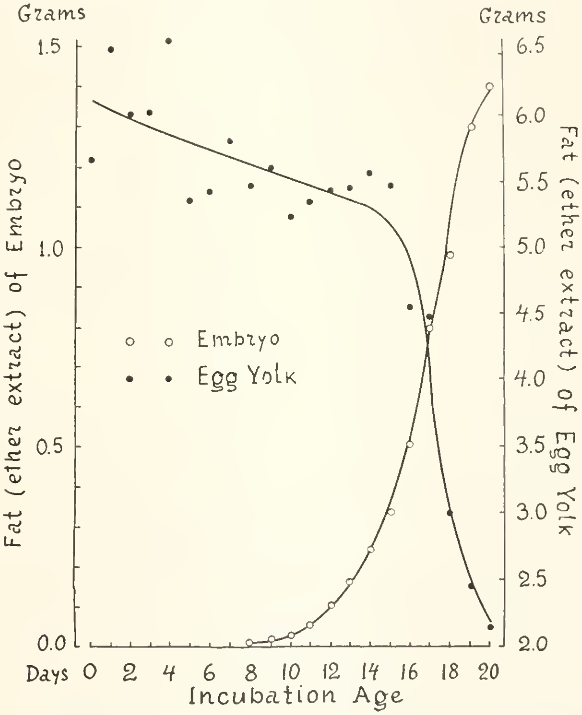

FIG. 1. Changes in the fat (ether extract) content of the embryo and egg yolk during incubation.

RESULTS AND DISCUSSIONS

It has been found that the fat content of a fresh fowl's egg is directly proportional to its size. For that reason the amounts of fat obtained from an egg by previous workers can hardly be compared, unless the data are expressed in percentages of dry or wet weight of the substance. Similar inconsistency is observed in the data on iodine value, saponification number and refractive index of the fat from an egg yolk (Table I). These dissimilarities might be accounted for by the lack of uniformity in material and methods, such as: the method of extraction, care of the extract, age of eggs and possibly seasonal quality of eggs.

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[1] Both saponification number and iodine value were determined after the methods given in *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, third edition, 1930, on pp. 321–322.

It is evident from the data of Table II that the egg yolk is the main source of fat to the developing embryo. In a fresh egg there is about 99.5 per cent of yolk fat and only 0.5 per cent of fat in the remainder of the egg, including albumen and shell with shell membranes. At the time of hatching the yolk sac still has the largest portion of reserve fat for the nutrition of the chick after hatching. Next in amount is the fat absorbed by the embryo. Then a small amount of fat is retained in the waste matter and in the shell with

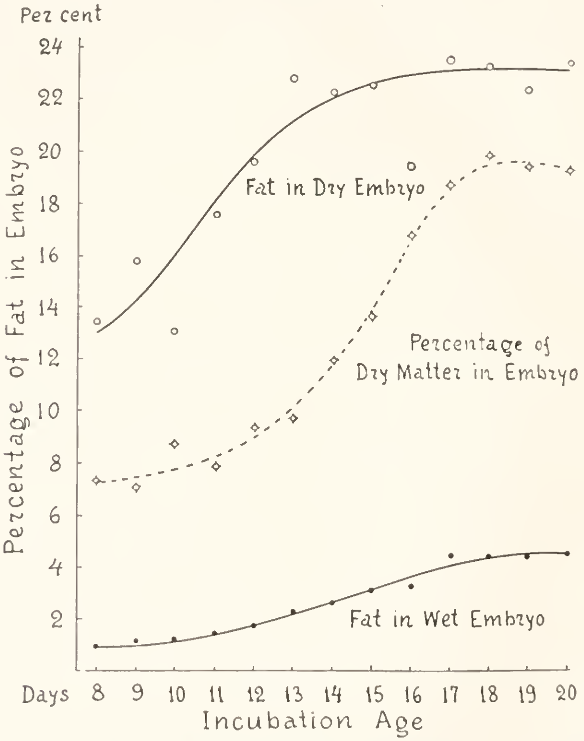

FIG. 2. Percentages of fat (ether extract) in the wet and dry weights of the embryo at various stages of incubation. (The curves of the above values are not identical due to changes in the dry matter content of the embryo, which is shown by dotted line.)

shell membranes. The combusted fat represents 33.61 per cent, or perhaps a larger amount if synthesis of fat had taken place during the incubation.

The daily fat content of the developing embryo and egg yolk (Tables III and IV) gives a general idea of the changes which go on within an egg during incubation (Fig. 1). The fat of the embryo increases noticeably only after about two weeks. The fat of the yolk at first decreases slowly; then it rapidly falls from about the sixteenth day up to hatching time.

The curves on the percentages of fat in the wet and dry embryo (Fig. 2) are not identical. Change in the moisture content of the embryo throughout the incubation period is the main influencing factor. Therefore a true percentage value should be taken from dry rather than wet weight of the embryo.

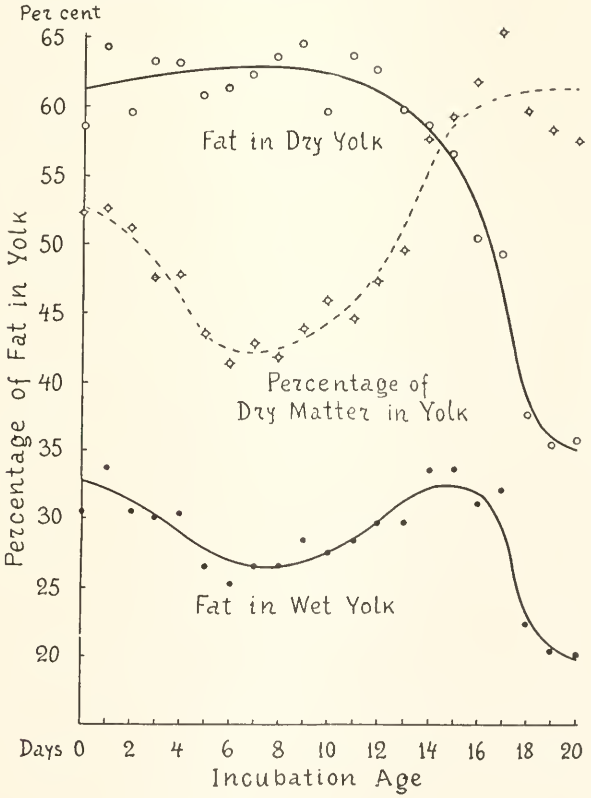

FIG. 3. Percentages of fat (ether extract) in the wet and dry weights of the egg yolk at various stages of incubation. (The curves of the above values are not identical due to changes in the dry matter content of the yolk, which is shown by dotted line.)

The influence of the moisture content of the egg yolk on the curves of the percentages of fat from wet and dry weight (Fig. 3) is still greater than the influence of the moisture content of the embryo. The curve of the percentage of fat in dry yolk is the only one which demonstrates regularity in changes of fat during incubation. This curve shows that the percentage of fat in yolk is increasing somewhat during the first week, declining during the second and dropping during the third week. The rise of the curve may be due to synthesis of fat from other chemical substances; the decline due to the noticeable growth of the embryo; and the drop due to the rapid and sole utilization of fat for growth and for energy supply to the embryo.

TABLE V

Refractive Index of Fat from the Chick Embryo and Yolk Sac

Stage of Incubation	Refractive Index $[(n)_D]$	
	Embryo Fat	Yolk Fat
days		
0	—	1.4685
9	1.4879	—
10	[1.4903]	—
11	1.4877	—
12	1.4841	—
13	1.4844	1.4687
14	1.4816	1.4695
15	1.4758	1.4693
16	1.4763	1.4695
17	1.4741	1.4675
18	1.4735	1.4691
19	1.4727	1.4696
20	1.4719	1.4690

The combusted fat accompanying embryonic development, is the expression of the energy expended. It has been observed that the greatest part of the egg fat is used toward the end of hatching time. The curve of the combusted fat would be very similar to the curve of the growth of the embryo (Romanoff, 1930).

The extracted fat from egg yolk throughout the incubation period shows hardly any change in the saponification and iodine numbers and refractive index, giving on an average 73.89, 190.69, and 1.4690 respectively. On the other hand, there was found a pronounced change in the iodine number and refractive index of the fat from the embryo (Table V, Fig. 4). The iodine number of the fat from the embryo at 16 days of incubation was 78.84; on the following days it

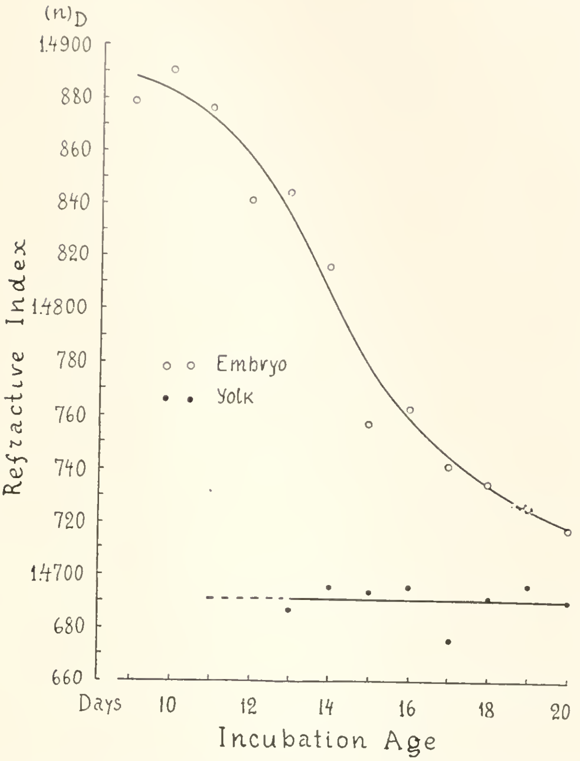

FIG. 4. Refractive index of the fat from the embryo and egg yolk during incubation.

was 82.03, 83.69, 84.03, 88.20, reaching 90.34 at the time of hatching. The refractive index was found to be high at the beginning of observation, nine days of embryonic age, and was rapidly decreasing towards hatching.

The author wishes to express his thanks to H. A. Faber for assistance in routine analytical work covering this paper.

SUMMARY

1. Fat content of fresh eggs is a subject of great variation, due primarily to disparity in size of individual eggs.

2. Iodine value, saponification number and refractive index of the fat from fresh eggs are rather constant, but only under a given experimental condition.

3. The main source of fat in an egg is the egg yolk, which furnishes the fat for nutrition and energy-supply of the developing embryo.

4. The amounts of fat in the growing embryo and egg yolk give a comprehensive idea about the changes in fat which go on within an egg during incubation.

5. The relative increase of fat in the embryo and the decrease of it in the yolk can be well demonstrated by the curves plotted from the data on the percentages of fat in dry weight.

6. The curve of combusted fat is quite similar to the curve of the growth of embryo.

7. Iodine value, saponification number and refractive index of the fat from the yolk sac of the developing egg are almost constant through the incubation period.

8. Iodine value and refractive index of the fat from the developing embryo are increasing and decreasing respectively during the latter part of incubation.

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IS THE ERYTHROCYTE PERMEABLE TO HYDROGEN IONS?

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I

The erythrocyte is generally believed to differ from most other cells in the ease with which its internal reaction is influenced by that of its surroundings. Even in the absence of so-called "penetrating" acids and bases such as CO_2, fatty acids, ammonia, etc., which alone are effective with ordinary cells, it is easy to bring about in it striking internal pH changes. The mere hydrogen ion concentration (strictly speaking, the hydrogen ion activity) of the surrounding medium, regardless of how it is produced, seems automatically to determine in the erythrocyte an internal hydrogen ion concentration that can be predicted by the principles of the Donnan equilibrium (Warburg, 1922; Van Slyke, Wu and McLean, 1923). This interrelation of external and internal pH is part of an important mechanism for preserving an approximately constant blood reaction under all ordinary physiological conditions.

While the establishment of a Donnan equilibrium between the erythrocyte and its surroundings is almost universally considered to be due to a passage of ions across the cell membrane, there has been some doubt as to the particular ions involved in the case of pH changes. Partly, no doubt, owing to our customary methods of measuring and defining the reactions of aqueous solutions, and partly to the fact that physiologists, in general, have had to deal more frequently with the penetration of cells by acids than by alkalies, it has been customary in the past to postulate a ready permeability of the erythrocyte to hydrogen ions. But it has been pointed out by Van Slyke, Wu and McLean (1923) and others that exactly the same end results would be obtained if the permeability were to hydroxyl rather than to hydrogen ions, since, as is well known, the relation existing between these two ions in aqueous solutions is such that the activity of the one is related in a fixed manner to that of the other.

In cases where our interest is primarily in equilibria, it is a matter of indifference whether the cell is permeable to the hydrogen or to the hydroxyl ion, or to both; indeed, by the study of equilibrium states alone it is impossible to reach any decision as to the mechanism by which these states have been reached. Since most of the work heretofore done on the erythrocyte has been concerned with equilibria, it is not surprising that the question of the relative penetrating powers of hydrogen and hydroxyl ions has received almost no attention. However, this question is obviously one of considerable importance to those interested primarily in the mechanism of cell permeability. The erythrocyte, as is well-known, appears to be permeable to anions and impermeable to cations (for a summary of the evidence see Jacobs, 1931), and a single exception to the general rule would make necessary a more complicated theory of ionic permeability than would otherwise be required. It is true that the hydrogen ion is unique in several other respects, and it is by no means inconceivable that it might be in its powers of penetrating the erythrocyte as well. It would, however, considerably simplify the situation if direct evidence could be furnished in favor of the other alternative which is, *a priori*, the more probable of the two.

In the present paper evidence of this sort has been obtained by the use of a simple method, which may, incidentally, prove to be useful in the investigation of other problems connected with the physiology of the erythrocyte. Though this evidence is not presented as conclusive proof of the view that pH adjustments between the erythrocyte and its surroundings are produced through the agency of the hydroxyl ion, it is believed that the observed facts may be explained more simply in this way than in any other; indeed, we have been unable to find any other simple and plausible explanation that even remotely fits the facts.

II

One of the most convenient methods for studying permeability to hydrogen and hydroxyl ions is to employ cells which contain natural indicators of some sort whose color is affected by changes in intracellular reaction. It happens that the erythrocyte belongs in the category of cells showing such color changes. As is well known, hemoglobin, in the presence of sufficiently high concentrations of acids, loses its red color and becomes converted into brownish, or in dilute solutions, yellowish acid hematin. The fact that this change is irreversible and is not associated with a definite pH value prevents it from being employed in exactly the same way as those of true indicators; but it may, nevertheless, when used with judgment, be of considerable usefulness. In the case of hemolyzed blood in which intimate contact of the acid with the hemoglobin is immediately insured, the color change in the presence of 0.15 M NaCl occurs practically instantly at pH values of 2.5 or lower; at pH 3.5 the time required may be several minutes and at pH 4.0 may be measured in hours. As far as can be determined by the rather crude methods available, there is no sharp upper limit at which the change entirely ceases.

The same color change occurs, but much more slowly, in the case of suspensions of unhemolyzed erythrocytes; and it is this fact that renders it useful as an indicator of the penetration of acid into the cells. Since, unfortunately, it does not take place sharply at any fixed pH value, it is not a highly accurate means of measuring acid penetration; but within certain limits it appears to be capable of being employed as at least an approximate measure of the rate of this process. The color changes with intact erythrocytes are naturally most conspicuous when the cells are sufficiently numerous to give a distinctly red color to the suspension. A mixture of 1 part of blood to 250–500 of solution (1 or 2 drops to 25 cc.) is that which we have found most suitable; with greater dilutions the color of the suspension is yellowish to begin with and the color change consequently less distinct; with higher concentrations of blood the effect on the pH of the solutions is too great. The color change may be made considerably more striking by first treating the erythrocyte suspension with a trace of carbon monoxide, but to avoid the possibility of unknown complications this procedure was not employed in the present experiments.

Under most conditions which bring about a change in the color of the cells, hemolysis also occurs. The question therefore arises whether the acid penetrates the cells and acts upon the hemoglobin within them or whether the reaction is rather an extracellular one with hemoglobin first liberated by hemolysis. We believe that the former alternative is probably the correct one, for two reasons. In the first place, careful observations have been made, by the method of Jacobs (1930*b*), of the time at which the first traces of hemolysis appear. This proves almost invariably to be after rather than before a distinct color change has occurred (see Tables I, II and III). In the second place, it has been found that within a certain pH range the presence of Na_2SO_4 in proper amounts may either prevent hemolysis almost completely or at least delay it for an hour or more without at all slowing the change of color. Indeed, by starting with a 10 per cent saccharose solution containing 0.015 N HCl, the addition of Na_2SO_4 to a concentration of 0.05 M may considerably accelerate the color change, while strongly retarding hemolysis. In one experiment the following figures were obtained:

	Color Change	Hemolysis
Saccharose alone	285 seconds	390 seconds
Saccharose + Na_2SO_4	165 seconds	>1 hour

The probable reason for the accelerating effect of Na_2SO_4 on the color change will be discussed below. That for the inhibition of hemolysis is not certainly known, but at all events such experiments furnish a clear demonstration of the possibility of intracellular color changes.

Though liberation of hemoglobin from the cells is not necessary in order that the reaction may occur, it must not be thought that the entrance of acid is not influenced by injury to the cells. In observing the behavior of a given suspension, it may be noted that the color change does not proceed gradually and fairly regularly, as it does in the case of hemolyzed blood at sufficiently high pH values, but that for a time the change is extremely slow or entirely invisible, and then suddenly becomes much more rapid, as if a barrier of some sort had been broken down by injury to the cell. Shortly after this point, hemolysis occurs. As contrasted with the slow rate at which the color change is produced by HCl, that in the presence of so-called "penetrating" acids, such as acetic or butyric acids at the same or even considerably less acid pH values, is strikingly rapid. In this respect the behavior of the erythrocyte is similar to that of ordinary cells containing natural or artificially introduced indicators.

In the experiments here reported the erythrocytes were obtained from defibrinated ox blood. The cells were not "washed," since it was believed to be less serious to introduce into the solutions employed the slight traces of blood proteins unavoidably present in dilutions of 1 : 250 or 1 : 500 than perhaps to change the fundamental properties of the erythrocytes in the manner described by Kerr (1929) by previously removing these proteins. The pH determinations were in all cases made with the quinhydrone electrode at the conclusion of each set of experiments. Though the addition of the blood somewhat reduced the acidity of the original solutions, the latter were present in such excess that the pH changes in most cases did not amount to more than a few tenths, or at the highest concentrations of acid, a few hundredths of a pH unit.

The color changes were determined in test tubes by eye, the time given being that at which a distinct color difference could be detected between the experimental tube and an appropriate control. Though it was found that a certain degree of refinement could be introduced by the use of a colorimeter, the advantages of this instrument for the present purposes were not sufficient to compensate for the considerably greater time required with it to carry out a series of experiments. The determinations of the time of hemolysis, as has already been mentioned, were made by the method of Jacobs (1930*b*), of necessity on a separate, but as nearly as possible identical, suspension of the same blood in the same solution. The few cases in which hemolysis appeared slightly to precede the color change are probably to be accounted for by the fact that the figures were obtained not from a single experiment but from two parallel experiments.

III

The method described in the preceding section was first employed by us in an attempt to throw further light upon an earlier observation (Jacobs, 1930*a*) that the rate of acid hemolysis is greatly influenced by the salt concentration of the external medium. Illustrations of this effect will be found in Table I and in Tables II and III which, taken together, show the same thing.

TABLE I

Effect on time of color change and of hemolysis of adding different amounts of NaCl to 0.02 M HCl in 0.3 M saccharose. All times are in seconds.

Concentration of NaCl	Color change	Beginning of hemolysis	75 per cent hemolysis
—	116	130	153
0.0002	118	127	147
0.0004	108	128	135
0.0008	104	—	—
0.0016	110	—	—
0.0031	116	—	—
0.0063	111	123	137
0.0125	102	111	125
0.025	87	95	119
0.05	71	80	92
0.1	60	71	81
0.2	45	72	80

The more rapid rate of acid hemolysis in the presence of NaCl might conceivably be due to a more ready penetration of the cells by the acid; on the other hand, since hemolysis is a complicated process, it is also possible that the salt might accelerate the destruction of the erythrocyte in some other way. It was in an attempt to decide between these two possibilities that advantage was taken of a method by which the penetration of the acid could be made directly visible. It was thought that in the presence of different concentrations of salt, a parallel between the time of color change and of hemolysis, with the color change preceding hemolysis, would indicate an effect of the salt on the actual rate of penetration of the acid. That such a parallel does in fact exist is shown by the experiments to which reference has already been made. In Table I, for example, it will be noted that concentrations of NaCl less than about 0.01 M have little or no effect on either process, but that at higher concentrations the acceleration of hemolysis very closely follows that of the color change.

The question now arises why the entrance of acid and the onset of hemolysis occur more quickly in the presence of NaCl than in its absence. Several conceivable explanations may immediately be dismissed as being decidedly improbable. For example, the case presents superficial analogies with the increased permeability of various cells in the presence of NaCl (Harvey, 1911; Osterhout, 1911, 1922, etc.); but it is easy to show that the accelerated entrance of acid into the erythrocyte and the subsequent hemolysis occur equally readily in the presence of pure isotonic $CaCl_2$ or of physiologically balanced mixtures of NaCl and $CaCl_2$. It has, in fact, been found that a considerable variety of salts are, without exception, effective in facilitating the entrance of HCl into the erythrocyte, though, as mentioned above, sulfates in certain concentrations may inhibit the subsequent hemolysis. It is very improbable, therefore, that there is any close connection between this phenomenon and the older

TABLE II

Time (in seconds except where otherwise indicated) of Color Change and of Hemolysis with Different Concentrations of HCl in 0.15 M NaCl

Concentration of HCl	Color change (hemolyzed blood)	Color change (unhemolyzed blood)	Beginning of hemolysis	75 per cent hemolysis	pH after hemolysis
0.08 N	Almost instantaneous	8	12	25	1.26
0.04	Almost instantaneous	25	29	35	1.55
0.02	Almost instantaneous	39	39	50	1.95
0.016	Almost instantaneous	42	33	60	1.95
0.008	Almost instantaneous	46	44	90	2.25
0.004	Almost instantaneous	53	60	145	2.61
0.002	3	57	72	194	3.06
0.001	9	72	75	232	3.78
0.0005	135	480	67(?)	232	4.62
0.0001	3-4 hours	∞	∞	∞	—

observations on the increase of permeability produced by sodium salts, which is a specific effect peculiar to these and perhaps a few other salts, and which is readily antagonized by calcium.

Another explanation, applying specifically to the erythrocyte, was next considered, namely, that in the presence of salts some constituent of the cell surface might be removed, thus rendering the cell more permeable to ions. In this connection reference may be made to the work of Brinkman and van Dam (1920), who have reported that it is easy to remove lecithin from the erythrocyte by solutions of electrolytes but not by those of non-electrolytes. An explanation of this sort was, however, rendered very improbable by a simple experiment which consisted in comparing the effectiveness of previous washings of the cells with isotonic NaCl solutions, with the actual presence of small quantities of the salt at the time of hemolysis. In one such experiment it was found that ox erythrocytes were no more readily hemolyzed in 0.3 M glycerol containing 0.015 N HCl after six previous washings in isotonic NaCl than before. If the observed effect were merely due to the removal of something from the cell surface, six washings ought to have been far more effective than was the presence in the solution at the time of hemolysis of as low a concentration of NaCl as 0.01 M or less; but this was not the case. From this and similar experiments the conclusion was drawn that it is necessary for

Table III

Time (in seconds except where otherwise indicated) of Color Change and of Hemolysis with Different Concentrations of HCl in 0.3 M Saccharose

Concentration of HCl	Color change (hemolyzed blood)	Color change (unhemolyzed blood)	Beginning of hemolysis	75 per cent hemolysis	pH after hemolysis
0.32 N	Almost instantly	12	31	52	0.82
0.16	Almost instantly	15	24	29	1.03
0.08	Almost instantly	25	30	36	1.29
0.04	Almost instantly	56	59	69	1.50
0.02	Almost instantly	90	132	144	1.70
0.01	Almost instantly	185	231	269	2.04
0.005	3	270	330	450	2.26
0.0025	75	720	960	—	2.56
0.0012	240	3 hours	4 hours	—	2.87
0.0006	2 hours	5 hours	—	—	3.30

the salt actually to be present with the HCl in order to exert its characteristic influence.

In an attempt to throw further light upon this question, systematic observations were made over a considerable range of acid concentrations both in the presence of NaCl (0.154 M) and in its absence, using for the latter purpose a 0.3 M solution of saccharose. Two such experiments, made on the same blood under as nearly as possible comparable conditions (except for a slight accidental difference, not believed to be significant, in the concentration of the blood in a few of the individual experiments) are summarized in Tables II and III.

The data contained in Tables II and III, which were obtained before the subject had received any theoretical treatment, seemed at first sight somewhat puzzling; but, as will be shown, they have proved to be capable of a very simple semi-quantitative explanation on the basis of a hypothetical permeability of the erythrocyte to hydroxyl rather than to hydrogen ions. It will be noted that in the experiments in question, as in previous ones, color changes and hemolysis always occurred more rapidly in the presence than in the absence of NaCl, other conditions being the same. But not only were the rates of color change and of hemolysis slower in solutions of saccharose than in those of NaCl but in the absence of salt both of these processes entirely failed to occur at concentrations of acid that were otherwise always effective. In other words, it would appear that the salt, in addition to its other effects, influences the position of final equilibrium of the system.

Another difference between the experiments represented in Tables II and III, whose meaning was at first far from clear but which we now believe to be of considerable theoretical significance, is that whereas in the non-electrolyte solutions, over a fairly wide range, the rate of color change and of hemolysis is roughly proportional to the concentration of acid (*i.e.*, doubling the concentration of acid approximately halves the time required for the attainment of the chosen end-point), in the NaCl solutions the concentration of acid is of much less importance. Thus, a forty-fold change in concentration (from 0.001 N to 0.04 N) is seen in Table II to decrease the time required for the color change only from 72 to 25 seconds and for the beginning of hemolysis from 75 to 29 seconds.

In an attempt to account for these various observations, we turned to a consideration of the conditions governing ionic exchanges of various sorts between the erythrocyte and its surroundings. Not only has this treatment of the problem furnished a plausible and simple explanation for all the facts mentioned above, but it has, in addition, apparently thrown some light upon the equally important question of the relative permeability of the erythrocyte to hydrogen and to hydroxyl ions.

IV

The passage of ions across a membrane can occur only in such a way that electrical neutrality is at all times preserved. Thus, Cl', an ion which is known to pass readily between the erythrocyte and its surroundings, might do so by being exchanged for another univalent anion such as HCO'_3 or OH' or, if the cell were permeable to $H^{\cdot}$ ions, it might cross the membrane in company with one of these cations. The absolute rate of movement of pairs of ions, either in the same or in opposite directions, is not known, but in any given case, by an obvious application of the mass law, the rate of total movement at a given instant ought to be proportional to the product of the concentrations (or activities) of the two members of the pair. When the final equilibrium is reached the total movements in the two directions must balance, giving, therefore, as the condition for equilibrium either:

$$[H]_{\text{solution}} \times [Cl]_{\text{solution}} = [H]_{\text{cell}} \times [Cl]_{\text{cell}}$$

or

$$[OH]_{\text{cell}} \times [Cl]_{\text{solution}} = [OH]_{\text{solution}} \times [Cl]_{\text{cell}}$$

as the case may be. These expressions are identical with those obtained by Donnan by more rigorous thermodynamic reasoning.

In the case under consideration, it is unfortunately impossible to follow the entire course of the diffusion process by which the final theoretical ionic equilibrium tends to be established. Indeed, in a case where a cell is being hemolyzed and its hemoglobin is at the same time undergoing a fundamental chemical change, anything like an equilibrium is unthinkable. The only part of the process, therefore, about which we can form a reasonably accurate conception is its initial stage when the cell and the surrounding medium both have known compositions. Though information limited merely to this stage is less than we might desire, it is better than none at all; and, indeed, it seems reasonable to suppose that the conditions that determine the initial rate of ionic exchange would exert a similar influence over much of the remainder of the process. It will therefore be instructive to calculate the values of the initial mass law effect for several different types of solutions.

In Fig. 1 are represented the concentrations (which will here be employed as an approximation in place of the more accurate activities, and which are expressed in mols per kilo of water) of certain ions at the instant when a normal erythrocyte is placed in a given solution

FIG. 1

Cell	Solution
(8×10^{-2}) Hb′	
(16×10^{-2}) $K^{\cdot}$	$Na^{\cdot}$ (y)
(8×10^{-2}) Cl′	Cl′ $\left(x+y-\frac{10^{-14}}{x}\right)$
(5×10^{-8}) $H^{\cdot}$	$H^{\cdot}$ (x)
(2×10^{-7}) OH′	OH′ $\left(\frac{10^{-14}}{x}\right)$

containing as its only salt NaCl and as its only acid HCl. The pH of the interior of the erythrocyte has been taken as 7.3 and for simplicity all of its salts are represented as KCl. The concentrations given for the cell are merely roughly approximate values for arterial blood (see Henderson, 1928, page 196), since for our present purposes, where only the order of magnitude of the results is significant, a higher degree of accuracy would be meaningless.

As regards the external solution, we have two necessary conditions:

$$[H] \times [OH] = K_w = \text{approximately } 10^{-14}$$

and

$$[Cl] + [OH] = [H] + [Na].$$

Knowing [H] and [Na] which are here represented by x and y, respectively, the values of [Cl] and [OH] are immediately determined by the equations just given. In cases involving sums (but not products) of ionic concentrations in acid solutions, [OH] is usually so small that it may be neglected.

Under the conditions represented in Fig. 1, the directions and initial relative rates of ionic movement, for the two possible methods of pH change, would be determined by:

$$[H]_{\text{solution}} \times [Cl]_{\text{solution}} - [H]_{\text{cell}} \times [Cl]_{\text{cell}}$$

and

$$[OH]_{\text{cell}} \times [Cl]_{\text{solution}} - [OH]_{\text{solution}} \times [Cl]_{\text{cell}}$$

respectively. Substituting in these equations the various concentrations given in Fig. 1, we have:

$$x^2 + xy - 10^{-14} - 4 \times 10^{-9} \qquad (1)$$

and

$$(2 \times 10^{-7})\left(x + y - \frac{10^{-14}}{x}\right) - \frac{8 \times 10^{-16}}{x}, \qquad (2)$$

from which we may calculate, as has been done in Table IV, the relative tendencies for the erythrocyte to become more acid (or less alkaline) in solutions of different degrees of acidity in the presence of an approximately physiological concentration (0.16 M) of NaCl and in its absence.

It is, of course, obvious that the figures in this table, taken singly, have no very great significance; and it is not even permissible at a given pH value to compare with each other the figures for hydrogen and for hydroxyl ions, since the actual rates of penetration of the cell by these ions will depend not merely on the mass law factors but upon the specific properties of the individual ions as well. But for each kind of ion separately over a series of different pH values, the figures have an important relative significance to which attention may now be directed.

It will be noted in Table IV that according to the hypothesis of permeability to hydrogen ions, the initial mass law factor, for the concentrations of acid actually employed in the experiments, should increase in the sodium chloride solution somewhat more rapidly than the concentration of hydrogen ions. In the saccharose solution, on the other hand, over the same range of concentrations the increase should be approximately proportional to the square of the hydrogen ion concentration. An examination of Tables II and III shows that in the salt solution the observed rate of penetration of the acid is only slightly affected by its own concentration, while in the saccharose solution it is roughly proportional to the first rather than to the second power of its concentration. Evidently the observed facts are in complete disagreement with the hypothesis of permeability to hydrogen ions.

TABLE IV

Mass law effect with 0.16 M NaCl and isotonic saccharose solutions at different pH values, for movement across the cell membrane (a) of H· with Cl′ and (b) of OH′ in exchange for Cl′. Internal conditions as described in the text.

pH	0.16 M NaCl		Isotonic saccharose	
	H· with Cl′	OH′ in exchange for Cl′	H· with Cl′	OH′ in exchange for Cl′
7.0	12.00×10^{-9}	24.00×10^{-9}	-4.00×10^{-9}	-8.00×10^{-9}
6.0	15.60×10^{-8}	31.20×10^{-9}	-4.00×10^{-9}	-8.00×10^{-10}
5.0	15.96×10^{-7}	31.92×10^{-9}	-3.99×10^{-9}	-7.80×10^{-11}
4.0	16.01×10^{-6}	32.01×10^{-9}	6.00×10^{-9}	1.20×10^{-11}
3.0	16.10×10^{-5}	32.20×10^{-9}	9.96×10^{-7}	1.99×10^{-10}
2.0	17.00×10^{-4}	34.00×10^{-9}	1.00×10^{-4}	2.00×10^{-9}
1.0	26.00×10^{-3}	52.00×10^{-9}	1.00×10^{-2}	2.00×10^{-8}

On the other hand, both in the presence and the absence of NaCl the rate of color change and of hemolysis are in good semi-quantitative agreement with the predicted mass law effects according to the hypothesis of permeability to hydroxyl ions. It is difficult to believe that this agreement is merely the result of chance. Admitting our ignorance of all but the probable beginning of the diffusion process, and making due allowance for the complicating effects of injury to the cells, especially in the more acid solutions, and the very rough nature of the calculations where so many simplifying assumptions have been made, it is nevertheless true that no other explanation of the facts has as yet been found which is at the same time so simple and so well in agreement with the other known properties of the erythrocyte.

The entire failure of color changes and of hemolysis to occur in sugar solutions at acid concentrations which readily bring them about in the presence of NaCl is a necessary consequence of the general theory of ionic exchanges. It will be noted in Table IV that for sugar solutions of pH 7.0, 6.0 and 5.0 the mass law factors for both mechanisms of ionic exchange have negative signs. That is to say, in such solutions the total movement of hydrogen or of hydroxyl ions, as the case may be, must be in the opposite direction from that found in the remaining solutions. In other words, erythrocytes in such solutions should theoretically become more alkaline rather than more acid.

This same problem may also be approached in a slightly different way by introducing the idea of a final equilibrium. Though it is obviously impossible, because of imperfect knowledge of the behavior of hemoglobin and of the erythrocyte at decidedly acid reactions, to calculate the final equilibrium conditions in cells exposed to any desired acid solution, it is nevertheless possible to determine what external solution would cause no internal pH change in normal cells. For the simplified erythrocyte already dealt with, either equation (1) or equation (2) (omitting quantities which are negligibly small) yields the following equation for equilibrium:

$$x^2 + xy = 4 \times 10^{-9}.$$

Solving for x, we have as the external hydrogen ion concentration in equilibrium with an internal pH value of 7.3:

$$x = \frac{\sqrt{y^2 + 1.6 \times 10^{-8}} - y}{2}. \qquad (3)$$

By means of this equation the pH values in Table V have been calculated.

It will be noted in this table that in the entire absence of salt the pH of equilibrium is 4.2, and therefore any solution less acid than this ought theoretically to cause the simplified erythrocyte to become more alkaline rather than more acid. For a concentration of NaCl of 0.0001 M, the critical pH is 4.5, and for one of 0.001, 5.4, etc. These figures, of course, cannot be expected to hold exactly in the case of actual erythrocytes where conditions are more complicated than those here considered. But the general principle itself, to which attention has already been directed by Netter (1928) and which has been put to a practical use by Bruch and Netter (1930), appears to be a sound one whose neglect has probably been responsible for considerable confusion in the past in experimental work with the erythrocyte.

TABLE V

External pH in equilibrium with an internal pH of 7.3 with various external concentrations of NaCl. Internal conditions as described in the text.

Concentration of NaCl (*mols. per liter*)	Equilibrium pH
—	4.2
0.0001	4.5
0.001	5.4
0.01	6.4
0.1	7.4

One further point about Tables II and III deserves mention. Not only is the color change of intact cells affected by the presence or absence of electrolytes, but a similar, though less marked, effect is observable in the case of hemolyzed blood. It is possible that we may here be dealing with a case similar to that reported by Adair, Barcroft, and Bock (1921), who found that even in blood hemolyzed by distilled water there is evidence that the cells, though invisible, may still be sufficiently well preserved to produce characteristic effects upon the dissociation curve of hemoglobin. It is not unreasonable, therefore, to expect that even in hemolyzed blood there might be some evidence of the same salt-acid effect that is found with intact cells. Whether this is a complete explanation of the observed facts, however, or whether some additional principle is involved cannot at present be stated with certainty.

SUMMARY

1. The penetration of acids into mammalian erythrocytes may be followed macroscopically by means of the color changes that occur when hemoglobin is converted into acid hematin.

2. The penetration of the acid precedes, rather than follows,

hemolysis. In certain cases, penetration may be observed without subsequent hemolysis.

3. Over a considerable pH range, both in the presence and in the absence of NaCl, the rate of acid penetration into the erythrocyte, as inferred from the time of color change, is in semi-quantitative agreement with that predicted for a system permeable to hydroxyl and not to hydrogen ions. There is an entire lack of agreement with the theoretical behavior of such a system when the permeability to the two ions is reversed. The simplest explanation of the observed facts is that the hydrogen ion, like other cations, is unable to enter the erythrocyte easily.

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ASCIDIANS OF THE BERMUDAS

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The ascidians of the Bermuda islands have already been described by Van Name (1902) and a revision published by the same author in 1921 under the title "The Ascidian Fauna of the West Indies." In view of this the present paper is confined to a brief account of the fauna as a whole, a description of several new species, and a few notes upon certain other forms.

The ascidians of the Bermudas are almost identical, species for species, with those of the West Indies and Gulf of Mexico, and there is little doubt that, together with the bulk of the Bermuda fauna, they have been carried there from those regions by the west Atlantic drift.

Like ascidians throughout the world, their habitat is determined as a compromise between two factors, their ability to withstand wave-action and exposure, and their need for a good flow of well oxygenated water. They may accordingly be divided into three groups, those to be found only under stones, those attached to the upper surfaces of stones, or to various corals and sea-fans, and those found beyond the outer reefs.

Apart from such classification of habitat further description such as specified localities is practically unnecessary. Species found under stones in one locality are likely to be found in similar positions throughout the islands. The same holds true for forms that are attached to exposed surfaces.

The material upon which this paper is based was obtained through the facilities of the Bermuda Biological Station, St. Georges, and also through the assistance of Louis Mowbray, Esq., of the Government Aquarium.

Fauna List

Aplidium (*Amaroucium*) *bermudae* (Van Name)	on corals
Aplidium (*Amaroucium*) *exile* (Van Name)	under stones
Trididemnum savignii (Herdman)	on algae, under stones
Trididemnum orbiculatum (Van Name)	under stones
Didemnum candidum Savigny	under stones, on corals
Didemnum (*Polysyncraton*) *amethysteum* (Van Name)	under stones
Leptoclinum macdonaldi (Herdman)	on corals, etc.
Lissoclinum fragile (Van Name)	under stones
Polycitor (*Eudistoma*) *olivaceus* (Van Name)	on stones, corals, etc.

Polycitor (*Eudistoma*) *convexus* (Van Name)	on corals, etc.
Polycitor (*Eudistoma*) *clarus* (Van Name)	on stones, corals, etc.
Polycitor (*Eudistoma*) *capsulatum* (Van Name)	on corals, etc.
Clavelina picta (Verrill)	on sea-fans, wrecks, etc.
Clavelina oblonga Herdman	under stones, on corals
Cystodytes dellechiaiae (Della Valle)	on corals, stones, etc.
Distaplia (*Holozoa*) *bermudensis* (Van Name)	under stones, on corals
Perophora viridis Verrill	under stones
Perophora bermudensis n.sp.	under stones
Ecteinascidia turbinata Herdman	on stones, corals, wrecks
Ecteinascidia conklini n.sp.	under stones
Ecteinascidia conklini var. *minuta*	under stones
Ascidia nigra Savigny	on stones, etc.
Ascidia curvata Traustedt	under stones
Botryllus schlosseri (Pallas)	under stones
Botrylloides niger (Herdman)	on and under stones
Symplegma viride Herdman	under stones
Polyandrocarpa (*Eusynstyela*) *tincta* (Van Name)	under stones
Polycarpa obtecta Traustedt	on stones, corals, reefs
Styela partita (Stimpson)	on stones, corals, reefs
Pyura vittata (Stimpson)	on stones
Microcosmus exasperatus Heller	under stones, on reefs

The Bermuda fauna is exceptionally rich in members of the family Perophoridæ. Two well-known forms, *Ecteinascidia turbinata* and *Perophora viridis*, are common. Their close relationship, however, is emphasized by the discovery of transitional types. These new forms may frequently be found growing under the same boulder as those two species.

Inasmuch as the existing descriptions of *Ecteinascidia turbinata* are of immature zoöids, a somewhat detailed account is made here of this species; such an account also forms a basis for comparison of the other forms.

Ecteinascidia turbinata Herdman.

This species was originally described by Herdman from material collected at Bermuda by the Challenger expedition. Large orange colonies develop attached to the upper surfaces of rocks where currents are relatively strong.

The essential organization of the zoöid is shown in Fig. 1. The structures shown here for the first time are the heart, and the gonads with their ducts. The heart is of the same relative size and length, and is in the same relative position as in species of Ascidia or Phallusia. The gonads develop in the intestinal loop on the left side, as in Perophora, the ovary being surrounded by a ring of testicular lobes. The vas deferens during its distal course follows the intestine and they open together some distance short of the atrial siphon. The oviduct, on the other hand, is relatively short and wide. It passes

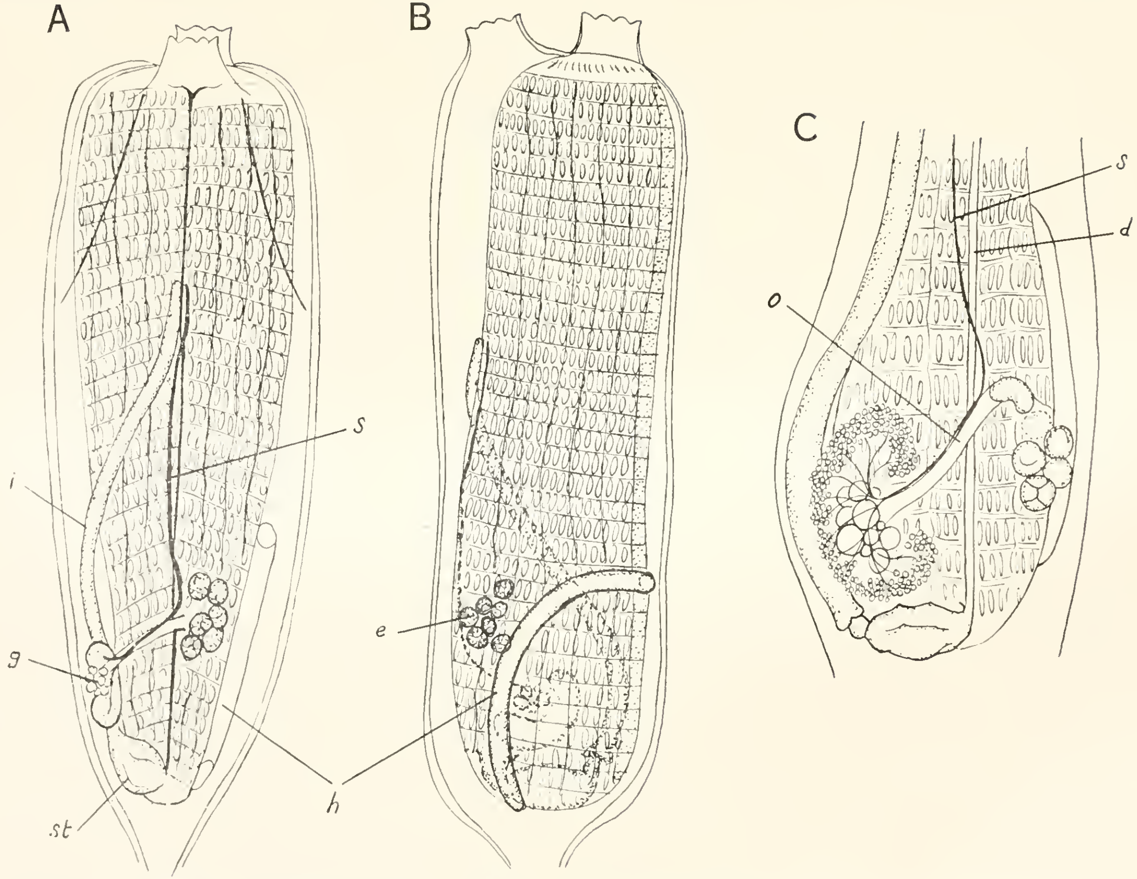

Fig. 1. *Ecteinascidia turbinata* Herdman. Mature blastozoöids, A, from dorsal, B, from right side; C, from dorsal and left aspect. *d*, dorsal lamina; *e*, embryos; *g*, gonads; *h*, heart; *i*, intestine; *o*, oviduct with egg emerging from it into right atrial cavity to join developing embryos; *s*, sperm-duct; *st*, stomach.

from the ovary to the dorsal lamina, opening beyond it into the atrial cavity of the right side. A single egg at a time is forced up the duct. During this brief passage it becomes greatly elongated through compression, rapidly regaining its spherical shape as it emerges.

The above features are generic, the characters of specific importance being as follows,—the tendency to grow on the exposed surfaces of rocks, the red or orange colour, the proximity of the atrial to the branchial siphon, the possession by the adult zoöid of about thirty rows of stigmata, and the formation of tadpole larvæ with twelve rows of stigmata. The breeding season extends from about June first to August or September.

Ecteinascidia conklini n. sp.

This species was at first mistaken for young colonies of *E. turbinata*. The generic features are identical with those of *E. turbinata*. The specific differences are the following: it is found only on the under side of rocks, the colour is green or yellow-green with but a thin red ring around the siphons, sometimes absent; the atrial siphon is long and placed some considerable distance from the branchial siphon; the rows of stigmata in the zoöids average about twenty in number, while smaller tadpoles are formed with but six rows of stigmata; the eggs are smaller, and the breeding season commences about one month later than that of *E. turbinata*, under identical conditions.

Ecteinascidia conklini var. *minuta* n. sp.

This form was found with mixed colonies of *E. conklini typica* and *Perophora viridis*. It differs from the type species in a few minor features only, which nevertheless seem worthy of recording.

It is smaller; the number of rows of stigmata in the zoöid rarely exceeds fifteen; the atrial siphon is short and close to the branchial siphon while both bear prominent ridges, and the eggs and larvæ are smaller although the tadpole possesses six rows of stigmata as in the other. The breeding season as far as is known coincides with that of *E. conklini typica*.

Probably the most definite feature distinguishing both *E. conklini typica* and *minuta* from *E. turbinata* is the curvature of the intestine in the former, tending to form a secondary loop. The intestine of *E. turbinata* is but very slightly curved in comparison.

Perophora bermudensis n. sp.

This species forms mixed colonies with the common *Perophora viridis*, both growing in great profusion on the under surface of stones where there is a good circulation of water.

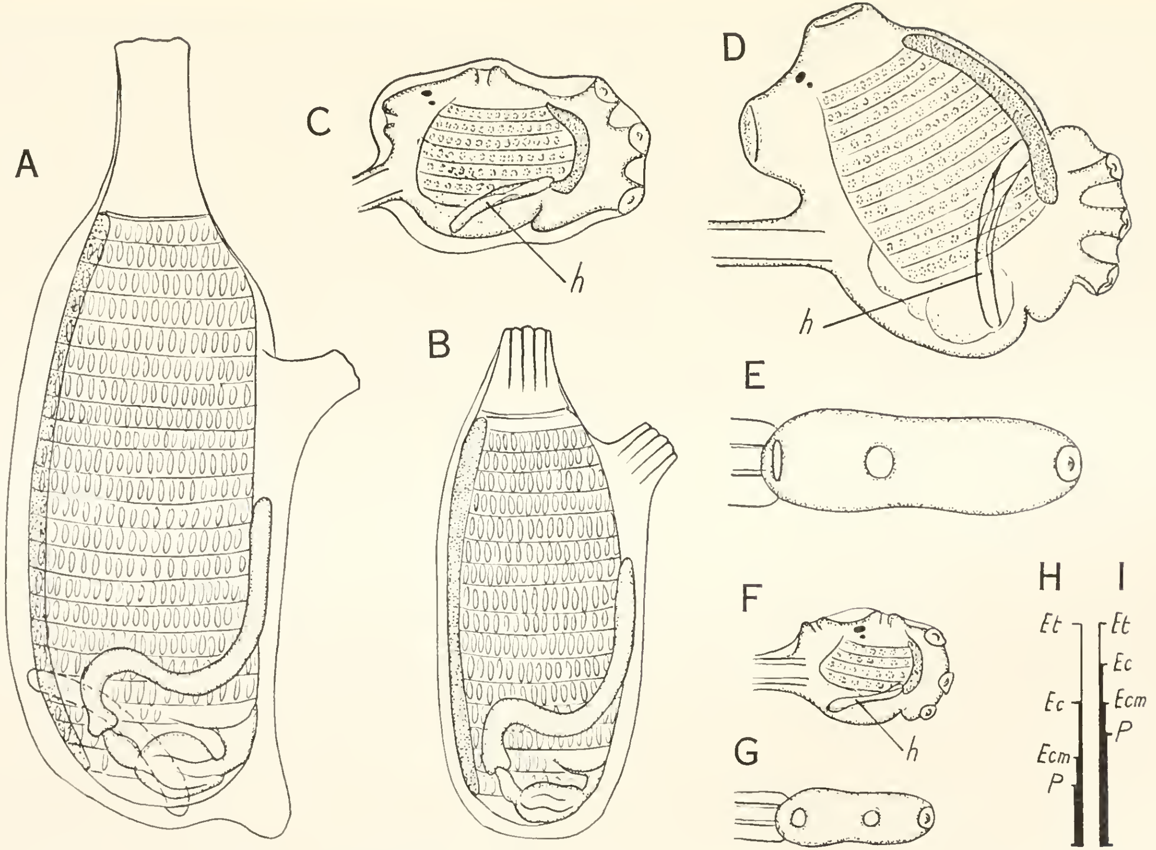

FIG. 2. A, blastozoöid of *Ecteinascidia conklini* n. sp., from right side. B, blastozoöid of *E. conklini* var. *minuta*, from right side. C, tadpole of *E. conklini* (that of *E. conklini minuta* is identical except for being smaller). D, tadpole of *E. turbinata* from side, E from top. F, tadpole of *Perophora viridis* from side, G from top. H, relative sizes of mature zoöids of the three species of Ecteinascidia and of Perophora. I, relative sizes of the eggs of the same forms.

The zoöids are drawn all to one and the same scale, while the tadpoles are also drawn to one scale.

h, heart.

Perophora viridis has a greenish tinge, as its name implies, and both oozoöid and blastozoöid have four rows of stigmata. *Perophora listeri* is similar in that the greenish colour is quite absent, but it forms by no means such dense colonies. *Perophora annectens* differs from both these species in that it has but three rows of stigmata.

Perophora bermudensis possesses blastozoöids with five rows of stigmata (although the oözoid has but four) and is accordingly unique. It may be readily distinguished from *P. viridis* with which it is usually entangled, by the absence of any green colour and the somewhat larger size of the zoöids.

Its structure is shown in Fig. 3, and it is seen that not only are there five rows of stigmata but that about a third of the stigmata of the first and second rows are common to both. This condition prevails in the smallest and youngest blastozoöids in which stigmata are discernible.

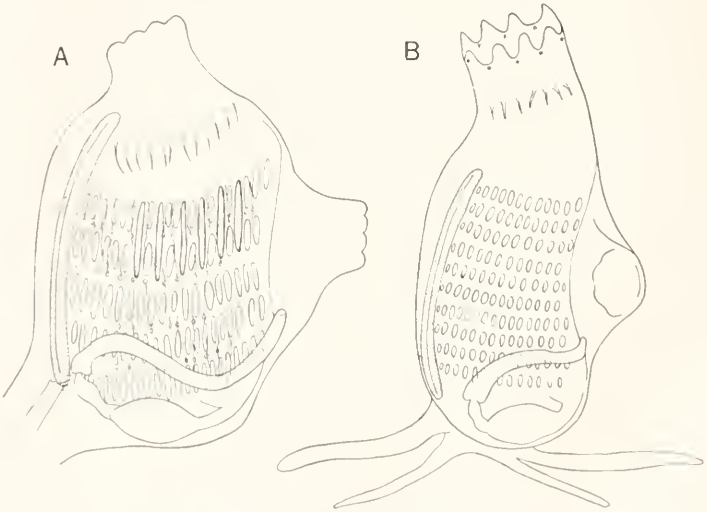

FIG. 3. A, blastozoöid of *Perophora bermudensis* n.sp. B, oozoöid of *Ecteinascidia conklini* or else a blastozoöid of *Perophoropsis herdmani* Lahille.

Perophoropsis herdmani Lahille.

This form was described by Lahille in 1890 from Banyuls and has not been rediscovered. It differs from species of *Perophora* in that there are at least ten rows of stigmata. It is a genus therefore intermediate between *Perophora* and *Ecteinascidia*.

At Bermuda, mixed with colonies of *Perophora viridis*, *P. bermudensis*, and *Ecteinascidia conklini*, were found individuals corresponding in structure to *Perophoropsis;* these are shown in Fig. 4.

There is, however, another possibility. It was determined in the case of *Ecteinascidia turbinata*, *E. conklini*, *Perophora bermudensis*, *Clavelina lepadiformis*, *Botryllus gigas*, and *Botrylloides leachii*, that in developing blastozoöids the number of rows of stigmata, while yet non-functional although perforate, equals or may slightly exceed the number of stigmata rows in the zoöid from which they arose. In no case was it less than that number. This statement is probably true for ascidians as a whole and will form the subject of further investigation.

This phenomenon accordingly rules out the possibility that the Perophoropsis-like forms were young blastozoöids of *Ecteinascidia;* while the fact that the oozoöid of *E. turbinata* possesses from the start twelve rows of stigmata makes it impossible that they are young oozoöids of that species. With *E. conklini* the case is different. The oozoöid first becomes active with but six rows of stigmata, and accordingly it is possible that the forms in question are slightly-grown individuals of this type, especially as they were solitary and without budding stolons, although occurring not far from one another. Another feature which supports such a contention is that the individual stigmata are but half the length of those of *Ecteinascidia* or *Perophora* species. Whether they are blastozoöids or oozoöids, they are probably but half-grown.

Provisionally, therefore, these perophorids will be assumed to be young oozoöids of *Ecteinascidia conklini* rather than the *Perophoropsis* of Lahille. If this is the case, then it should be noted that additional rows of stigmata appear in the oozoöid when the stigmata of the original six rows are merely a fourth of full adult size, and cannot be distinguished from those first six rows.

Genus *Clavelina.*

There is some confusion as to the number of species of *Clavelina* to be found in the Bermuda and West Indian region.

From material taken on the Challenger expedition at Bermuda Herdman described a form that he named *Clavelina oblonga*. In 1900 Verrill, in his general account of the fauna of the Bermudas, described an ascidian under the name *Diazona picta*. In the account of the Bermuda ascidians published by Van Name in 1902 this last was changed to *Rhodozona picta* on the ground that it differed materially from the type of *Diazona* and was intermediate in character between *Diazona* and *Clavelina*. In his later account (1921) of the ascidian fauna of the West Indies Van Name includes both these forms under *Clavelina oblonga*, the *Diazona picta* of Verrill being considered to be large colonies and Herdman's *Clavelina oblonga* young colonies of one and the same species. This he describes as a form in which the zoöids of a young colony are almost completely separated from one another but which in older colonies become enclosed for their greater part in a common gelatinous test.

Such a conclusion is believed to be the result of two factors, the study mainly of preserved material, and of material collected not later than May, that is, of material somewhat immature. Preservation destroys pigmentation and in these two forms results in a tremendous shrinkage of the thoracic part of the zoöids.

These forms are believed to be both clavelinids but also to be quite distinct species. On this assumption then the one should be known as *Clavelina oblonga* Herdman and the other as *Clavelina picta* (Verrill). It is hoped that the following descriptions will show this to be the case.

Clavelina oblonga Herdman.	**Clavelina picta (Verrill).**
Habitat.	*Habitat.*
Attached to the under-surface of stones near low-water level, very rarely in more exposed positions.	Attached to sea-fans, corals, submerged wrecks, etc., from low-water level to a few fathoms; never found under stones; a good flow of water seems to be necessary.
Breeding season.	*Breeding season.*
End of April until August.	End of June until August or September.
Form of colony.	*Form of colony.*
Number of zoöids rarely exceeds 40, usually much less. They are attached to a basal stolon, but otherwise are separated from one another. Budding occurs throughout breeding season so that zoöids of all sizes are to be found.	Colonies usually possess more than 40 zoöids and may have as many as 1000. The zoöids are embedded in a common test, which is usually divided into corms. During the summer the thorax of each zoöid extends beyond the common test, at other times it is usually completely embedded. Budding does not occur during the breeding season or the months preceding it and so the size of the zoöids is very uniform in any one colony.
Pigmentation.	*Pigmentation.*
Test crystal-clear. Branchial sac with flecks of white pigment near its anterior end. Abdominal region yellowish. In living state whole colony perfectly transparent.	Test unpigmented but slightly opaque. Endostyle, dorsal lamina, and peripharyngeal bands densely coloured with purple or carmine cells. They also extend throughout the abdomen and then accumulate in the ends of the test vessels. They appear in the late embryo in such places and the oozoöid is as highly coloured as the blastozoöids.
Egg and tadpole size.	*Egg and tadpole size.*
0.31 mm. diameter (egg). 2.25 mm. total length (tadpole).	0.49 mm. diameter (egg). 3.30 mm. total length (tadpole).
Post-larval development.	*Post-larval development.*
Tadpole and young oozoöid have from the first, two rows of definitive stigmata.	Tadpole and young oozoöid have from the first, four rows of definitive stigmata. All other species of *Clavelina* have but two.

Clavelina oblonga usually occurs in groups of two or three during May and increases in number throughout the breeding season until about forty zoöids of various size may be found in one colony. This was found to be the case in both 1930 and 1931, and the implication is that the oozoöid forms a few winter statoblasts which regenerate and give rise to the type of colony just described during the following spring, while such a colony dies off at the end of the summer, failing to form a second generation of statoblasts. That is, the life-cycle is completed within 18 months; otherwise, large colonies should be found similar to those of *Clavelina lepadiformis*, which is not the case.

That *Clavelina picta* was originally taken to be a diazonid is due to a superficial similarity in type of budding. In *Diazona violacea* budding by abdominal strobilation occurs after the breeding season has ended, and only then. So that every autumn each zoöid forms eight or ten compact bodies which regenerate slowly through the winter and spring to become sexually mature in the late summer; the process is then repeated. Therefore the zoöids are uniform in size and remain embedded in the common test, although the anterior part of the thorax of each extends separately during the late summer.

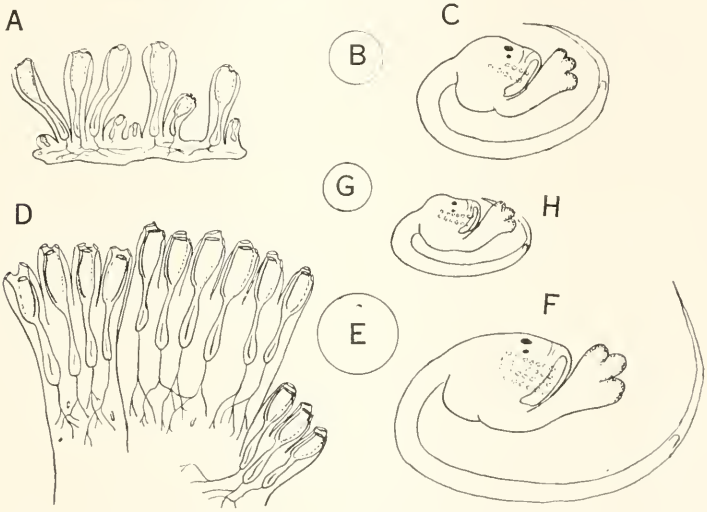

FIG. 4. A, colony of *Clavelina oblonga* Herdman. B, C, egg and tadpole of same. D, part of a colony of *Clavelina picta* (Verrill). E, F, egg and tadpole of same. G, H, egg and tadpole of *Clavelina lepadiformis*.

The eggs and tadpoles are drawn to the same scale.

In *Clavelina picta* winter statoblasts are formed after the breeding season and apparently at no other time. The oozoöid probably forms about ten such bodies. These regenerate, grow and become sexually mature during the winter and spring. They carry on these processes close together within the test of the oozoöid, and so remain within a common test. During the late summer these processes are repeated, resulting in the formation during the following year of a colony composed of about ten corms, each containing ten or more zoöids. This may be repeated a third year to form massive colonies. During maturity the anterior parts of the zoöids extend from the common test just as in *Diazona*, and as in *Diazona* they contract and degenerate after the breeding season while the thoracic extensions of the test are sloughed off. Thus, apart from the difference in budding, strobilation of the abdomen in *Diazona* and the formation of post-abdominal extensions in *Clavelina*, the cycle is much the same in the two genera and it is understandable that a superficial similarity results. The separation of the zoöids in large colonies of *Clavelina lepadiformis* in contrast to the condition in *C. picta* is due probably to the very thin test of the former, so thin that when the statoblasts grow into new individuals they form independent vertical extensions of it from the first.

Budding in these forms will be the subject of a much more detailed investigation.

Symplegma viride Herdman.

This species was described first by Herdman from material collected at Bermuda by the Challenger expedition, and has since been discovered throughout the West Indies, and in the East Indies from the Philippines to the Red Sea. A full description with illustration of a zoöid is given by Van Name (1921).

This form has at various times been included in the Styelidæ and in the Botryllidæ. A few additional observations made at Bermuda merely emphasize its relationship to both these groups. In fact it so completely bridges the gap, both in its adult structure and organization and in its development, between these two families that their maintenance as distinct families is an unnatural classification. It is accordingly proposed that the Botryllidæ be subordinated within the Styelidæ, either as a sub-family or merely as constituent genera.

The method of budding in its fundamentals is identical in the Polystyelidæ, Symplegma, and Botryllidæ.

Symplegma resembles polystyelids such as *Polyandrocarpa*, *Distomus*, or *Stolonica* inasmuch as each zoöid has its own atrial siphon, common cloacal cavities not being formed. Its general anatomy resembles that of *Polyandrocarpa* or *Polycarpa* except that its individuals are much smaller. Correlated with this reduction is a diminution in number of polycarps (to a single polycarp) and in number of rows of stigmata. Its structure differs from that of botryllids only in the absence of common cloacal cavities, and consequently in the lack of obvious systems in the colony. In other words, common cloacal cavities and the arrangement of zoöids into systems are the only major features that separate the botryllids from other styelids. Apart from these two characters *Symplegma* is so similar to *Botryllus* and *Botrylloides* that it exhibits the same degree of variation in colour and form of whole colonies. These varieties are even more definite and constant in *Symplegma* than in the Botryllidæ, the commoner colour varieties being,—green, green and white, green and brown, black and white, and orange. With the exception of the last, all colonies are very compact and sheet-like as in botryllids, the adult zoöids being pressed together, young buds appearing in large numbers together with clusters of ampullæ at the colony margin. The orange variety, however, is different. Instead of the budding stolons being very short, they are even longer than in *Stolonica socialis*, with the result that the zoöids are often widely separated from each other while the individual zoöids assume a comparatively vertical position. It may, in view of this difference, be worth recognizing the orange

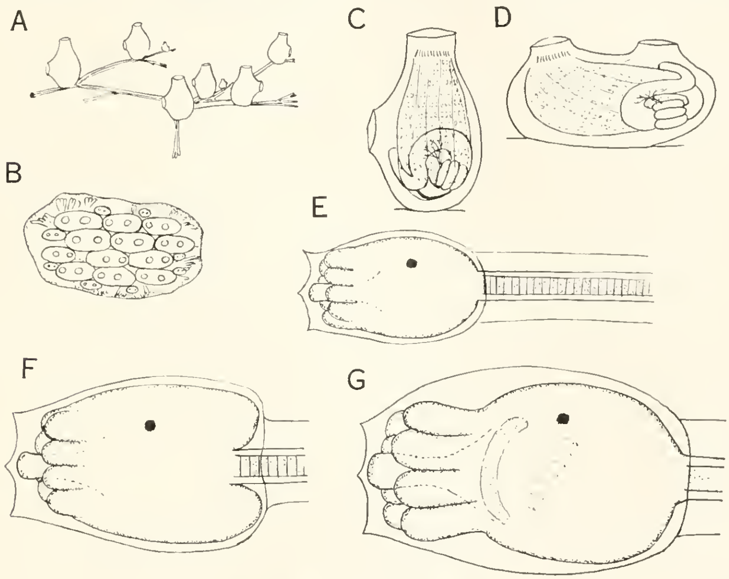

FIG. 5. A, colony of *Symplegma viride stolonica* n.v. B, colony of *Symplegma viride typica*. C, zoöid of *stolonica*. D, zoöid of *typica*. E, F, G, tadpoles of *Symplegma viride*, *Distomus variolosus*, and *Botryllus gigas*, respectively, drawn to the same scale.

form as a definite variety. If so, it is suggested that it be named *Symplegma viride stolonica*.

The essential unity of such forms as *Symplegma*, *Botryllus*, and *Distomus* is perhaps best shown in the study of the development. The tadpole of *Symplegma* is almost identical with those of *Distomus*, *Stolonica*, and *Styelopsis*, except that it is somewhat smaller. It has the same degree of organization; it has the same single but composite sense organ (probably developed from the otolith after the primitive eye was lost, an otolith and eye being found only in *Styela* itself); and in *Symplegma* and *Distomus* there is a long anterior mental process surrounded by a ring of ampullæ. *Botryllus* and *Botrylloides* both produce tadpoles with a similar mental process and ring of ampullæ, there being eight ampullæ in each of these genera and also in *Symplegma*. While the botryllid tadpoles are the more highly organized, the young oozoöids developing from these and from tadpoles of *Symplegma* are hardly to be distinguished.

Altogether there are greater differences between a genus such as *Styela* and the polystyelids than there are between these last and the botryllids.

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THE LIFE HISTORY OF EPIBDELLA MELLENI MACCALLUM 1927, A MONOGENETIC TREMATODE PARASITIC ON MARINE FISHES

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At present our knowledge concerning the life histories of the members of the order Monogena is extremely scanty. Although the life histories of the monogenetic trematodes are apparently much simpler than those of the digenetic forms, they have been less extensively studied, and definite information concerning them is, with a few exceptions, fragmentary. Of the sub-order Monopisthodiscinea Fuhrmann the only life history known is that of *Gyrodactylus* (Kathariner, 1904). Of the sub-order Monopisthocotylinea Odhner, which includes *Epibdella*, no life histories have been reported. In the sub-order Polyopisthocotylinea Odhner two life histories, those of *Polystomum integerrimum* (Zeller, 1872, 1876) and *Diplozoön paradoxum* (Zeller, 1872*a*), have been reported. The dimorphic development of *Polystomum integerrimum* on the gills of the tadpole and in the urinary bladder of the frog as given by Zeller (1872) has been questioned by several workers, including Stunkard (1917) who states that "the findings of Zeller are so unusual that one is led strongly to suspect he confused two different species." This leaves our definite knowledge of monogenetic life histories limited to two genera, *Gyrodactylus* and *Diplozoön*. The present investigation of *Epibdella melleni* MacCallum 1927 presents the third definitely-known life history of the Monogena and the first of the sub-order Monopisthocotylinea.

The adult of *Epibdella melleni* was first described by MacCallum (1927) as parasitic on the Pacific puffer (*Spheroides annulatus*), the spadefish (*Chaetodipterus faber*), and various species of angel fishes (*Angelicthys* and *Pomacanthus*) from the New York Aquarium. Due to the necessarily closed salt water system of the Aquarium, the infection spread rapidly to all tanks which contained susceptible fishes, and for several years it has been a very grave source of danger to all susceptible fishes.

This study was undertaken at the suggestion of Mr. Charles M. Breder of the New York Aquarium and Professor H. W. Stunkard of

New York University. The writers wish to thank Mr. Breder for his constant aid in the collection of material, for the identification of the fishes, and for information regarding their susceptibility, and Professor Stunkard for his helpful suggestions during the preparation of the manuscript. This study was made possible by a grant of the New York Zoölogical Society in the form of a Research Fellowship which was held by the senior author during the summer of 1930 and by the junior author during the academic year 1930–31.

MATERIAL AND METHODS

All material used for this study was obtained from infected fishes at the New York Aquarium. Adult specimens were obtained by scraping the body and cornea of the fish with a scalpel. In this way considerable mucus was obtained along with the parasites. This material was placed in small stender dishes, covered with sea water, and allowed to stand for ten minutes or longer. It was found that the parasites became firmly attached to the bottom of the dish, and that the mucus material could easily be removed with a pipette. After several changes of sea water the organisms, attached to the dish, could be studied with the aid of a binocular. In this way observations of the process of egg-laying were possible. It was found that the organisms remained attached to the dish after fixation. Therefore they were fixed, stained, destained, and cleared while attached by changing the fluid in the stender dish. They were then removed to a slide with the aid of a pipette and covered with balsam. Schaudinn's fluid and saturated aqueous solution of mercuric chloride plus five per cent acetic acid were used as fixatives. Whole mounts were stained with paracarmine, and sections with Delafield's hæmatoxylin and erythrosin.

Larvæ were obtained by collecting eggs and allowing them to hatch. Whenever larvæ were wanted, adult worms were collected and allowed to remain in stender dishes. The worms rarely lived more than twenty-four hours, but during this time numerous eggs were usually laid. These were removed to another dish in which the water was changed several times a day. At the end of five to eight days the eggs hatched, and the larvæ could be isolated with the aid of a mouth-controlled pipette.

MORPHOLOGY OF THE ADULT

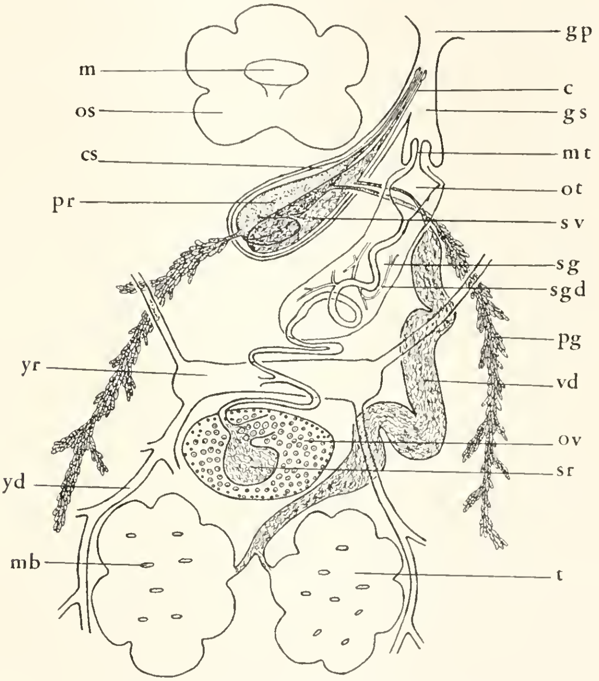
The adult of *Epibdella melleni* was described in some detail by MacCallum (1927), and this original description is substantiated by most of the observations of the present authors. The principal differences between the description as given by MacCallum and the present material lies in the details of the reproductive system and in the presence of fourteen larval or accessory hooks in the posterior sucker.

The adult of *E. melleni* (Fig. 1) varies from 3.5 to 5 mm. in length. The worms are usually white but sometimes contain numerous small patches of yellow pigment, and the covering of the body is smooth and unarmed. Two pairs of eyes are present near the anterior end of the body. The adhesive organs consist of one large posterior sucker and two smaller anterior suckers, all located on the ventral side of the body. The posterior sucker is round, smooth, and very shallow. It is 1.2 mm. in diameter in 4.5 mm. specimens and bears three pairs of large spines but no papillæ. The most anterior pair of spines is about .22 mm. in length, is slightly forked at the base, and is directed anteriorly, while the other two pairs, more posterior in position, arise posteriorly but are recurved in such a manner as to form veritable hooks whose points are directed anteriorly. The middle pair is the largest and most powerful, measuring about .30 mm. in length; the most posterior pair is much smaller, being only about .12 mm. in length, and its recurved points are relatively fine. There are also fourteen very small (10 μ in length) larval or accessory hooks arranged radially close to the margin of the sucker. These hooks are actually present on the larva and are apparently homologous with the larval hooklets of other monogenetic trematodes. Each of these hooklets is branched, and the two points are opposed (Fig. 13). The margin of the posterior sucker bears a secondary ring of very thin muscular tissue which serves to make the closure of the sucker on the substratum more secure and certain (Fig. 3). The anterior suckers are unarmed and bear no extra peripheral ring of muscular tissue. Both arcuate and dorso-ventral muscle fibers may be seen in sections of the suckers, but the greater portion of the suckers is made up of dorso-ventral fibers.

The oral sucker is large and powerful and is located in the median plane behind the anterior pair. The sucker is almost completely covered by thin lip-like structures which are a continuation of the dermal and epithelial layers of the ventral body wall (Fig. 2). These form a flat pouch whose opening coincides with that of the mouth sucker. Apparently these lips must be retracted when the sucker is attached to the substratum. When closed the oral sucker is pentalobate as shown in Fig. 1. It opens directly into the short œsophagus; there is no muscular pharynx. From the œsophagus arise the two main lateral intestinal trunks which have branches from practically all parts of the body. Each lateral intestinal trunk receives about fourteen smaller branches, and each of these smaller branches is further subdivided. No anastomosis of the branches could be definitely traced in sections, but MacCallum (1927) has described anastomoses of the branches in the median posterior region of the body. However, since anastomosis of the digestive cæca is quite common in other genera of the Monogena, it is possible that it occurs in this species.

The nervous system is composed of a crescent-shaped ganglionic mass anterior to the oral sucker, and two pairs of longitudinal nerve trunks, one of which lies just ventral to the main excretory channels, and the other of which lies more laterad (Fig. 1). The four eyes are embedded in the anterior ganglionic mass. The two trunks on each side are united to each other by three cross-connections, and they fuse in the posterior region of the body so that only two instead of four trunks enter the posterior sucker. In the posterior sucker these two trunks turn mediad and unite, forming a virtual 'ring' system, composed of two pairs of longitudinal trunks united anteriorly by two ganglionic masses and posteriorly by a connection in the posterior sucker. Anteriorly from the anterior ganglionic mass there arise two short trunks which innervate the anterior suckers, and these trunks also are united by a cross-connection. The smaller nerves branch off from the longitudinal trunks and from the cross-connection in the posterior sucker, which seems to serve as a nerve plexus for the numerous small branches which innervate the sucker. In some sections it appeared as if there were another cross-connection, semicircular in shape, in the posterior sucker besides the one shown in the figure, but this could not be traced with certainty.

The excretory pores are two in number and open to the dorsal side of the body, laterad to the posterior margin of the mouth. The two main excretory channels lead posteriorly from the excretory pores and unite near the posterior margin of the body. Slightly posterior to the excretory pores these channels widen to form the excretory vesicles which are very noticeable in the living adult. The longitudinal channels receive many branches, and these branches are repeatedly bifurcated in such a manner that the terminal ends ramify to all regions of the posterior two-thirds of the organism. Anteriorly from each excretory vesicle arises another longitudinal channel which passes forward, is greatly bifurcated, and curves mediad between the oral and anterior suckers. These two anterior channels unite in the median line behind the eyes so that the four main channels form virtually a 'ring' system composed of longitudinal channels united close to the anterior and posterior ends of the worm. The main channels in the body and also in the posterior sucker are identical in position and in connections with those of the larva which will be described later. The excretory system of the adult is obviously an elaboration of the pattern present in the larva, but the details of the system of the adult have not been traced. It is considered doubtful, however, whether there are four main longitudinal channels posterior to the excretory vesicles as described by MacCallum (1927), and it is thought probable that in his description the nervous system has been confused with the excretory.



TEXT FIG. 1. Semi-diagrammatic drawing of reproductive system of *Epibdella melleni.* Abbreviations: *c*, cirrus; *cs*, cirrus sac; *gs*, genital sinus; *m*, mouth; *mb*, muscular band through the testis; *mt*, metraterm; *od*, oviduct; *os*, oral sucker; *ot*, oötype; *ov*, ovary; *pg*, prostate gland; *pr*, prostatic reservoir; *sg*, 'shell gland'; *sgd*, 'shell gland' duct; *sr*, seminal receptacle; *sv*, seminal vesicle; *t*, testis, *vd*, vas deferens; *yd*, vitelline duct; *yr*, yolk reservoir.

The male reproductive system (text figure 1) is rather unusual in several respects. There are two testes (approximately .4 by .5 mm. in 4.5 mm. specimens) which lie in the mid-portion of the body; they are almost round in general outline with a lobate margin as seen from the dorsal or ventral surfaces, the amount of lobulation apparently increasing with the size of the specimen. They are not smooth bodies as described by MacCallum (1927). The testes of adult specimens are pierced by two to ten bands of muscle tissue which extend through the organs in a dorso-ventral direction. Their presence indicates that embryologically the testes might have been formed by the fusion of numerous small testicular bodies. Apparently the contraction of the muscles would force the contents of the testes into the short vasa efferentia which unite to form one very large sinuous vas deferens which passes forward on the left of the ovary. At the level of the expanded portion of the oötype the vas deferens is constricted and turns dorsad and mediad to enter the seminal vesicle. The ejaculatory apparatus consists of a very large pear-shaped cirrus sac located approximately in the median line directly posterior to the mouth; the smaller end of the organ tapers to open into the genital sinus to the left of the mouth. The cirrus sac is divided by a partition into two pyriform sacs: one is the seminal vesicle which receives spermatozoa from the vas deferens entering at the anterior end of the bulbous portion; the other is the prostatic reservoir which receives the prostate secretion. The prostate consists of two long, narrow, slightly branching, acinous glands; the duct of one enters the cirrus sac at its anterior end, and of the other at the anterior end of the bulbous portion at the same level as does the vas deferens. The posterior ends of the seminal vesicle and of the prostatic reservoir are folded back upon themselves in some of the specimens examined. The cirrus is a long, slender, heavy-walled tube continuous with the wall of the cirrus sac. It is divided internally by a septum continuous with its outer wall, thus forming two separate channels which extend to the end of the organ. One of these channels is continuous with the seminal vesicle and the other with the prostatic reservoir. The prostatic secretion stains deeply with erythrosin, and in sections or whole mounts gives a deep red color to the prostate glands, to their ducts, and to the prostatic reservoir. The secretion appears granular in stained sections and hyaline in living specimens. The cirrus is unarmed, but the end is marked off by a slight stricture. The cirrus sac opens into the genital sinus which is divided by a septum into two portions, a medial which receives the cirrus and a lateral into which the metraterm opens.

The female reproductive system of *E. melleni* (text figure 1) is quite simple in outline but has an unusual feature in the position of the seminal receptacle. The ovary lies in the mid-portion of the body, in the median plane, just anterior to the testes. It is oval and measures about .30 by .22 mm. in 4.5 mm. specimens. The seminal receptacle is definitely within the ovary and can be demonstrated as such in cross, sagittal and frontal sections, thus obviating the possibility that it is above or below the ovary. It may be clearly seen to contain spermatozoa when observed in sections with an oil immersion lens. The oviduct begins near the center of the ovary and is connected close to its origin with the seminal receptacle. Since both spermatozoa and ripe eggs are found in the initial portion of the oviduct and in the anterior part of the seminal receptacle, it is believed that fertilization takes place in this region. The oviduct passes out of the ventro-anterior portion of the ovary and arches dorsad and to the left. It then turns ventrad and crosses under the yolk reservoir from which it receives the yolk duct. The oviduct then continues tortuously to the oötype. The oötype is a long, slender, very muscular, coiled structure with a greatly enlarged tetrahedral anterior end. A uterus is absent, and the oötype opens via the metraterm into the lateral division of the genital sinus. The oötype is surrounded by a rather extensive 'shell gland' embedded in loose connective tissue. The ducts from the 'shell gland' unite to form several common tubules which open into the posterior portion of the oötype. A vagina and genito-intestinal canal are absent, and the vitellaria fill most of the interstitial space between the other organs throughout the body of the worm.

The Process of Egg Laying

In the living adult the yolk reservoir and the vitelline ducts may easily be distinguished with the aid of a low-powered binocular microscope, especially when they are full of yolk cells which appear as large gray structures against the clear body of the organism. When an egg is to be formed, a seemingly large quantity of yolk material streams from the yolk reservoir into the oötype, and it is driven back and forth by a rapid reversible peristaltic movement of this structure. The greater mass of yolk comes to lie in the anterior tetrahedral end of the oötype. During this time the shell material must lie around the yolk, and it is visible as a hyaline covering of the filament of the egg. The filament is at first filled with a central core of yolk material. This yolk material, due to peristaltic movements of the surrounding oötype, is driven forward into the tetrahedral region of the oötype, and further contraction of the posterior region of the oötype results in the shaping of the filament which consists at this stage of only hyaline material. During this time the yolk material at the anterior end of the egg is being shaped into its characteristic tetrahedral form. As the formation of the filament is completed the shell material may be distinguished around the body of the egg. The surging movement of the yolk continues until the egg is completely shaped, and then the shell material begins to harden. As it hardens it becomes yellowish, and this color gradually becomes deeper. When formation of the egg is complete, the anterior suckers, if attached, release their hold on the substratum, and the anterior end of the organism curves dorsad. The egg is then very quickly shot out from the anteriorly directed genital pore. The filament sometimes remains caught in the genital opening, but more often the egg is thrown completely free of the mother organism. After elimination of the egg, the shell material becomes a very deep yellow-bronze and is quite opaque.

Usually within a few seconds after the discharge of an egg, more yolk material flows from the yolk reservoir into the oötype, and the formation of another egg is begun. The whole process of egg formation requires from two to ten minutes, and the average time is about five minutes. The time-controlling factor seems to be the rapidity with which yolk material may be supplied to the yolk reservoir. Sometimes the yolk reservoir becomes completely emptied, and the process of egg-laying is stopped for a few minutes until the reservoir is refilled from the vitelline ducts. In some few organisms which seemed to lack sufficient shell material, the eggs were incompletely formed. In such cases the shell material usually formed the filament and the posterior portion of the tetrahedron, and the yolk material was shot out in a stream and was followed by the filament at the time the egg would have been released had it been complete (Fig. 12).

THE EGG

The egg of *Epibdella melleni* is tetrahedral in form and usually bears a single long filament and two shorter filaments with curved ends which form veritable hooks (Fig. 8). However, a number of eggs have been observed without hooks (Fig. 9), and others have been seen with three hooks and no filament. All of these types of eggs have been observed to come from the same adult, thus eliminating the possibility that they were eggs of different species. The size of the body of the egg is somewhat variable, but usually measures about .13 mm. on the edges of the tetrahedron. The filament is from .8 to 1.2 mm. in length and is sometimes seen to contain small ellipsoidal cavities throughout part of its length. The length of the filament, the presence or absence of and length of hooks, and the number of cavities in the long filament seem to be determined by the amount and distribution of shell material in the oötype at the time the egg is shaped. In some instances slightly larger and somewhat globiform eggs are produced (Fig. 11). These seem to result from a lack of sufficient shell material or from the presence of too much yolk in the oötype at the time of egg formation. The normal eggs are yellow in color when formed but turn to a golden bronze shortly after they are laid.

Hatching of the Egg, and Behavior of the Larva

Eggs of *Epibdella melleni*, isolated into small stender dishes in which the sea water is changed daily, hatch at room temperature in five to eight days. On the fourth to sixth days the larvæ may be seen within the eggs, and if an egg is broken open the larva is found apparently completely formed but non-motile. Just before hatching, the larva may be seen squirming about within the shell. A small circular opening is formed in the rounded corner of the egg, that is, on the corner which bears neither filament nor hook, but a preformed operculum was not observed. The larva emerges anterior end first. The anterior ciliated portion is thrust out, and the cilia meanwhile beat very rapidly. The larva may remain in this position for fifteen minutes. Then the beating of the cilia slowly pulls the organism out until only the posterior sucker remains within the shell. At the end of another fifteen minutes the larva is usually completely emerged. However, the time required for hatching is presumably affected by numerous undetermined factors. The larva, after emergence, swims very rapidly through the water, pausing only momentarily now and then on the bottom of the dish or on solid objects in the water. Within six hours, if the larva does not become attached to a suitable host, it apparently becomes exhausted, the rate of movement decreases, and the organism finally settles to the bottom, capable of only a slow crawling movement. This is presumably due to a loss of the ciliated epithelium, since such larvæ are always almost completely deciliated. Up to this time the posterior sucker does not appear to be functional.

Morphology of the Larva

The ciliated larva of *Epibdella melleni* (Fig. 4) is approximately 225 microns in length and 60 microns in width. It is slightly flattened dorso-ventrally in the anterior region, and, otherwise, is fusiform in general shape except for a constriction in the region of the mouth. The posterior third of the larva is composed of the posterior sucker which, at the time of hatching and for some time afterward, remains folded in such a manner as to be non-functional as a sucker. The anterior two-thirds of the larva is composed of what becomes the body of the adult, and it is, in general structure, similar to that of the adult except that the digestive and excretory systems are simple and the reproductive systems are not yet developed. The oral sucker is round and muscular and is located in the anterior part of the middle third of the body proper. It opens into a very short œsophagus which leads into two relatively large digestive cæca. These continue latero-posteriorly almost to the end of the body. No diverticula are present. Anterior to the mouth on the dorsal surface are two pairs of eyes. These are cup-shaped masses of pigment from the cavity of which protrude spherical hyaline lenses. Each of the posterior pair of eyes is approximately 16 microns in diameter and is directed anterio-laterad. Those of anterior pair are 12 microns in diameter and are directed posterio-laterad. The anterior suckers are not distinct sucking disks but are pad-like muscular areas, easily distinguishable in the living form.

The excretory system is composed of two relatively large excretory vesicles which are located slightly posterior and laterad to the mouth, four ducts which lead from them, and ten pairs of flame cells. The vesicles are quite distinct in the living organism and appear as large vacuolar structures. The excretory system opens by dorsal pores located on either side of the mouth as in the adult. The excretory system is shown diagrammatically in Fig. 7. Two large excretory channels extend, one from each vesicle, posteriorly into the posterior sucker. They are joined with each other by a cross-channel at the posterior end of the body proper. Each of these channels gives off an anteriorly directed branch which ends in a single flame cell about halfway between the vesicle and the cross-channel. The longitudinal channels continue into the sucker where each branches and ends in five flame cells as shown in Fig. 7. Leading anteriorly from the excretory vesicles is another pair of channels, one from each vesicle. These unite anterior to the mouth to form one median channel which continues forward between the eyes and branches laterally, and then each branch bifurcates anterio-posteriorly and ends in flame cells. In the region of the mouth are four flame cells, one on each side of the anterio-lateral and posterio-lateral sides of the oral sucker. The channels of these cells are probably branches of the anterior longitudinal channels, but this could not be determined with certainty due to the thickness of the oral sucker and the proximity of the ducts to it. These supposed connections are shown in Fig. 7. The arrangement of the excretory ducts of the larva is virtually a 'ring' system with two lateral excretory pores. The longitudinal channels and cross-connections of the larva have been checked in older specimens and have been found to be the main excretory channels of the adult.

The organism bears cilia in the anterior, middle and posterior regions. The anterior ciliated region extends from the anterior pair of eyes forward, and cilia cover practically all of the anterior region except the sucking pads. The middle ciliated region, extending from the posterior edge of the excretory vesicles almost to the posterior end of the body proper, is covered with cilia on the lateral, latero-dorsal, and latero-ventral surfaces. No cilia were seen on the mid-dorsal and mid-ventral surfaces in this region. The posterior ciliated region includes the lateral and dorsal surfaces of the posterior two-thirds of the posterior sucker. The cilia are relatively long and arise from an epithelial layer which can be seen distinctly in living specimens. This epithelial layer contains many large highly refractile granules of unknown function. When cilia are lost, the epithelial layer from which they arise is shed with them.

The posterior sucker, as folded when the larva is free-swimming, contains the definitive spines characteristic of the adult. These lie in a longitudinal position with the curved ends directed mediad as seen from the ventral surface. At the margin of the folded sucker, and lying ventrad to the definitive spines, may be seen the accessory or larval hooks. These are all the same size and measure approximately 10 microns in length. A single hooklet is shown in Fig. 13.

When maintained under a sealed coverslip, the ciliated larva may be seen to lose its cilia and to assume a shape and position more characteristic of the adult. The anterior sucking pads are usually very active in fresh preparations; these are stretched forward and are attached to the slide, and the body may be drawn afterward. The organism is capable of moving about in this fashion as well as by the use of the cilia. After this type of movement has been continued for some time, the ciliated epithelium begins to slough off, leaving areas of the normally ciliated region devoid of cilia. Apparently the first regions to become deciliated are those in the vicinity of the anterior sucking pads and those which cover the posterior sucker. However, the other ciliated epithelium is shed shortly afterward. If free-swimming larvæ are selected at random and examined, many are found which do not bear their full quota of cilia due to the sloughing of the epithelium. This may give rise to considerable confusion concerning the normal distribution, and it was necessary to examine a number of specimens in order to obtain the distribution shown in Fig. 4. Concomitantly with the sloughing of the ciliated epithelium, the posterior sucker is unfolded. As it is spread out, the definitive spines turn upon their longitudinal axis so that the curved ends are directed laterad, and the accessory hooks are pointed radially around the margin of the sucker. At this time the posterior sucker is functional and becomes firmly attached to the slide. Such an organism is shown in Fig. 5. This drawing was made of a larva which was isolated when ciliated and which had undergone the above transformations in about forty-five minutes while under observation.

In an attempt to interpret these transformations in relation to the normal life history of the organism, it is assumed that the larva is free-swimming until it comes in contact with a susceptible fish. Attachment is first by means of the anterior sucking pads. Then the cilia are lost, and the posterior sucker is unfolded, and a firmer attachment is afforded by means of the fourteen accessory hooks of the posterior sucker, aided perhaps by the most posterior pair of definitive spines.

Development of the Attached Form

After attachment the first morphological change to be noticed is the development of the anterior sucking pads into definite suckers. Then the mouth, which is round in the ciliated form, becomes lobate in outline as is that of the adult, and the digestive cæca show signs of becoming diverticulated. The posterior sucker becomes slightly larger in proportion to the size of the body, and the large spines increase in size and change in shape. A specimen in this stage that was obtained from an infested fish is shown in Fig. 6. No increase in size of the larval or accessory hooks could be noted, even when measurements of these hooks in young larvæ and in adult organisms were compared. All accessory hooks measured were between 9.8 μ and 10.7 μ in length. For this reason it is believed that these hooks are not of especial importance in the adult.

Further development of the organism seems to involve principally a further bifurcation of the digestive cæca, an elaboration of the excretory pattern, and the development of the reproductive system. The relatively large amount of space occupied by the reproductive system in the adult is responsible for the great differences in general appearance of the young (Fig. 6) and of the adult forms.

The sizes of the eyes change very little during the growth of the organism. Measurements made of the eyes of newly attached larvæ and of adult specimens showed no significant difference in size. The more anterior pair of eyes was found to be 10–13 μ in diameter, and the posterior pair was 15–18 μ in both young and adult specimens. However, the posterior pair of eyes in the adult is directed anteriomediad instead of anterio-laterad as in the larva. The eyes of the free-swimming larvæ are quite symmetrical in outline as shown in Fig. 4. Those of the adults are usually not spherical but are flattened in one direction or another. The eyes of some adults even appear conical with the base of the cone adjacent to the lens.

During the growth of the individual the relative amounts of growth of the three pairs of spines are not equal. The middle and the more anterior pairs grow at a rate that is relatively about twice that of the most posterior pair. Thus, the sizes of the first two pairs in the adult are over six times that of the same pairs in the youngest attached form, while the size of the most posterior pair is only three times that of the young form. Table I shows the average measurements of the three pairs of spines in young, medium-sized (about 1.5 mm.), and adult (over 3.5 mm.) specimens, and also the ratio of the sizes of the spines in the various sized specimens, the youngest forms being used as unity. The figures are the averages of the measurements of six specimens, and all three pairs were measured on the same individual. Therefore the ratios are strictly comparable.

OCCURRENCE AND PATHOGENICITY

When the adult of *Epibdella melleni*, obtained from the tanks of the New York Aquarium, was first described by MacCallum (1927), it was stated that the infection was probably introduced by a Pacific puffer (*Spheroides annulatus*) from California. However, it is the belief of Mr. Breder and his associates that there was no Pacific puffer in the Aquarium for some time preceding the discovery of the parasite and that the parasite was originally introduced and is being continually reintroduced with shipments of fishes from Key West and Nassau. Furthermore, the parasite occurs in both the Chicago and Philadelphia Aquariums, neither of which has Pacific fishes. Also, it is known that *E. melleni* will not survive very long exposure to the acid water of New York harbor and that it will multiply rapidly in the neutral tank water of the Aquarium. For many years no attempt was made to control the chemical composition of the water in the tanks of the New York Aquarium, and only after the installation of an efficient means of chemical control did the parasites become numerous, probably due to the very high acidity of the water previous to that time. For these reasons it is believed that the parasite is a West

TABLE I

	Average size of spines in mm.			Ratio of the size of the spine to that of the young attached form		
Pair of spines	Anterior	Middle	Posterior	Anterior	Middle	Posterior
Young	.035	.045	.038	1.0	1.0	1.0
Medium	.109	.174	.086	3.1	3.9	2.3
Adult	.216	.297	.118	6.2	6.6	3.0

Indian species and that it might have been continually present in small numbers in the New York Aquarium for many years before its discovery in 1927.

The fishes which have been found to be susceptible to infection with *Epibdella melleni* are as follows:

- Subclass Teleostomi
  - Order Acanthopteri
    - Family Carangidæ
      - *Caranx crysos* (Mitchill), Runner (X)
      - *Caranx hippos* (Linnaeus), Common Jack (X)
      - *Naucrates ductor* Linnaeus, Pilot Fish
      - *Trachinotus carolinus* (Linnaeus), Common Pompano
      - *Trachinotus glaucus* (Bloch), Old Wife or Palometa
      - *Vomer setapinnis* (Mitchill), Moonfish
    - Family Pomatomidæ
      - *Pomatomus saltatrix* (Linnaeus), Bluefish
    - Family Serranidæ
      - *Centropristus striatus* (Linnaeus), Common Sea Bass (X)
      - *Dermatolepis punctatus* Gill, Spotted Grouper (Pacific) (X)
      - *Epinephelus adscensionis* (Osbeck), Rock Hind
      - *Epinephelus guttatus* (Linnaeus), Red Hind
      - *Epinephelus morio* (Cuvier and Valenciennes), Red Grouper (X)
      - *Epinephelus striatus* (Bloch), Nassau Grouper (X)
      - *Paralabrax maculatofasciatus* (Steindachner), Spotted Cabrilla (Pacific)
      - *Promicrops itaiara* (Lichtenstein), Jewfish
    - Family Lutianidæ
      - *Lutianus analis* (Cuvier and Valenciennes), Muttonfish
      - *Lutianus apodus* (Walbaum), Schoolmaster
      - *Lutianus jocu* (Bloch and Schneider), Dog Snapper
      - *Lutianus synagris* (Linnaeus), Spot Snapper (X)
    - Family Hæmulidæ
      - *Anisotremus surinamensis* (Bloch), Black Margate
      - *Anisotremus virginicus* (Linnaeus), Porkfish
      - *Hæmulon album* Cuvier and Valenciennes, Margate
    - Family Sciænidæ
      - *Menticirrhus saxatilis* (Bloch and Schneider), Kingfish
      - *Micropogon undulatus* (Linnaeus), Croaker
    - Family Labridæ
      - *Lachnolaimus maximus* (Walbaum), Hogfish
      - *Tautoga onitis* (Linnaeus), Tautog
    - Family Ephippidæ
      - *Chætodipterus faber* (Broussonet), Spadefish
    - Family Chætodontidæ
      - *Angelichthys ciliaris* (Linnaeus), Queen Angelfish
      - *Angelichthys isabelita* Jordan and Ritter, Blue Angelfish
      - *Angelichthys townsendi* Nichols and Mowbray, Townsend's Angelfish
      - *Chætodon ocellatus* Bloch, Common Butterfly Fish
      - *Pomacanthus arcuatus* (Linnaeus), Black Angelfish
      - *Pomacanthus paru* (Bloch), French Angelfish
    - Family Acanthuridæ
      - *Acanthurus cæruleus* Bloch and Schneider, Blue Tang
      - *Acanthurus hepatus* (Linnaeus), Brown Tang or Doctor Fish
    - Family Balistidæ
      - *Balistes vetula* Linnaeus, Queen Triggerfish
      - *Melichthys bispinosus* Gilbert, Pacific Black Trigger (X)

Family Monacanthidæ
    Ceratacanthus schœpfii (Walbaum), Orange Filefish
    Stephanolepis hispidus (Linnaeus), Common Filefish
Family Ostraciidæ
    Lactophrys tricornis (Linnaeus), Cowfish
    Lactophrys trigonus (Linnaeus), Common Trunkfish
    Lactophrys triqueter (Linnaeus), Smooth Trunkfish
Family Tetradontidæ
    Spheroides annulatus (Jenyns) Pacific Puffer (X)
    Spheroides maculatus (Bloch and Schneider), Common Puffer or Northern Swellfish
Family Diodontidæ
    Diodon hystrix Linnaeus, Porcupine Fish
Family Triglidæ
    Prionotus evolans (Linnaeus), Striped Sea Robin
Family Malacanthidæ
    Malacanthus plumeri (Bloch), Sandfish

The species marked "X" seems to have developed a partial immunity after a short period of susceptibility. Most of these species are usually present in the tanks of the Aquarium, and newly arrived specimens always show a marked susceptibility to infection. However, after being present in the tanks for several weeks these species seldom show a slight and never a serious infection although they are continually exposed to reinfection. Some of the other susceptible fishes (*e.g.*, *Chætodipterus faber*, the spadefish) seemingly retain their infections, continually become reinfected, and die if they do not receive treatment. The central members of the spiny-rayed fishes (Acanthopteri), especially members of the families Serranidæ and Lutianidæ, are extremely susceptible, and the possibility of the development of an immunity seems to be more strongly suggested in these families although it is not shown by all members. The other species showing a distinct susceptibility are rather scattered phylogenetically but are all within this order.

Epibdella melleni has never (prior to June 1931) been observed on any of the following fishes, all of which have been continually exposed to infection while at the Aquarium:

Subclass Elasmobranchii
    Order Asterospondyli
        Family Ginglymostomidæ
            Ginglymostoma cirratum (Bonnaterre), Nurse Shark
        Family Galeidæ
            Mustelus canis (Mitchill), Smooth Dogfish
        Family Carchariidæ
            Carcharias littoralis (Mitchill), Sand Shark
    Order Batoidei
        Family Rajidæ
            Raja eglanteria Lacépède, Clear-nosed Skate

Family Dasyatidæ
Dasyatis centrura (Mitchill), Northern Sting Ray
Family Aëtobatidæ
Rhinoptera quadriloba (Le Sueur), Cow-nosed Ray

Subclass Teleostomi
Order Apodes
Family Murænidæ
Gymnothorax funebris Ranzani, Green Moray
Gymnothorax moringa (Cuvier), Spotted Moray
Order Haplomi
Family Pœciliidæ
Fundulus heteroclitus (Linnaeus), Common Killifish
Fundulus majalis (Walbaum), Striped Killifish
Order Lophobranchii
Family Syngnathidæ
Hippocampus hudsonius De Kay, Northern Sea Horse
Order Acanthopteri
Family Serranidæ
Mycteroperca bonaci (Poey), Black Grouper
Mycteroperca microlepis (Goode and Bean), Gag
Roccus lineatus (Bloch), Striped Bass
Family Lutianidæ
Evoplites viridis (Valenciennes), Blue-striped Snapper (Pacific)
Lutianus griseus (Linnaeus), Gray Snapper
Family Hæmulidæ
Hæmulon sp., Grunts
Family Sparidæ
Archosargus pourtalesii (Steindachner), Pacific Salema
Archosargus probatocephalus (Walbaum), Sheepshead
Family Sciænidæ
Leiostomus xanthurus Lacépède, Spot
Pogonias cromis (Linnaeus), Black or Sea Drum
Family Pomacentridæ
Pomacentrus rectifrænum (Gill), Pacific Beau Gregory
Family Diodontidæ
Chilomycterus schœpfii (Walbaum), Spiny Boxfish
Family Echeneidæ
Echeneis naucrates Linnaeus, Shark Sucker
Family Batrachoididæ
Opsanus tau (Linnaeus), Toadfish

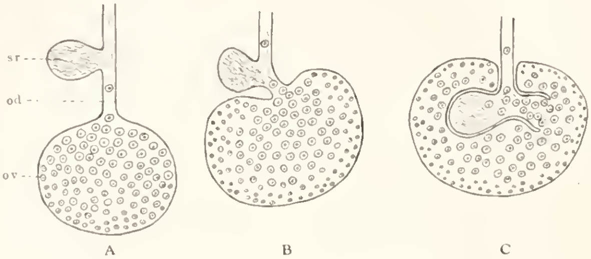
It is to be noted that although six elasmobranchs, representing six different families, were exposed, not one was ever observed to be infected. Also, five orders of the teleosts are represented on the list of non-susceptible fishes, and only one order, the Acanthopteri, is represented on the list of susceptible fishes. Therefore it seems as if all susceptible fishes may belong to the order Acanthopteri. Of this order a high susceptibility is shown by members of the families Serranidæ, Lutianidæ, and Ephippidæ, and no infections have been observed in the families Sparidæ, Pomocentridæ, Echeneidæ, and Batrachoididæ. Some of the other families are represented by members on both the susceptible and supposedly non-susceptible lists, and others are represented on the susceptible list only.

The injury produced to a susceptible fish is usually quite considerable, and if the infection is not treated, death often results. The trematodes attach themselves to the epidermis and to the conjunctiva, and in some cases they have been found in the gill and nasal cavities. Young specimens may be found on almost any part of the epidermis, but in some species (*e.g.*, *Chætodipterus faber* and *Promicrops itaiara*) the adults seem to be concentrated on the eyes. This concentration may be brought about either by the migration of young forms to the eyes, or by a high mortality rate of those which have become attached to the epidermis, or by both of these possible factors. The relative ease with which nourishment could be obtained from the soft tissue of the conjuctiva as compared with the difficulties encountered with the firmer epithelium between the network of scales seems to offer an obvious explanation of why such a concentration might occur. In mild infections the cornea is attacked and sometimes destroyed. If the infection is not treated, destruction of the eye follows, probably due to the effects of both *Epibdella* and secondary bacterial invaders. In very heavy infections the epidermis may suffer such severe injury as to cause the falling off of scales and the exposure of large areas of connective and muscular tissues with subsequent death of the fish. In one case, that of a Galapagos labroid which had escaped attention for some time, over two thousand adult worms were removed from the entire surface of the body. In the case of another similar infection several thousand eggs were found in the gill and nasal cavities. Both of these severe infections resulted in the death of the fishes.

Discussion

The life history of *Epibdella melleni* differs widely from the other definitely-known life histories of the Monogena. The life history of *E. melleni* seems to offer no homologies to the polyembryonic or pædogenetic condition found in *Gyrodactylus* (Kathariner, 1904) or to the characteristic fusion of the diporpa larvæ of *Diplozoön* (Zeller, 1872*a*). The direct development of a ciliated larva into the adult seems to be more closely related to the type of development described for *Polystomum integerrimum* (Zeller, 1872, 1876). However, the dimorphic development described for this species is probably, as pointed out by previous workers (*e.g.*, Stunkard, 1917), the result of the confusion of the life histories of two different species. It seems probable that the ciliated larva described by Zeller may develop into one of the two forms which he describes as the adult. If the development is similar to that found in *Epibdella*, it is quite probable that the ciliated larva of Zeller developed into the adult which was found on the gills of the tadpole. Also, if the ciliated larva of Zeller is shown to be the larva of the form in the urinary bladder, it is possible that a comparison of the two cases of direct development may still be drawn.

The wide differences between the life histories of *Epibdella* and *Gyrodactylus* seem to have an important bearing on the taxonomic grouping of the members of the order. Fuhrmann (1928) removed from the suborder Monopisthocotylinea Odhner the three families Protogyrodactylidæ, Gyrodactylidæ, and Calceostomidæ and created for them a new suborder, Monopisthodiscinea, of which the distinguishing characteristic was the absence of a definite posterior sucker and the possession of a posterior, armed, adhesive disk. A comparison of the life histories of *Gyrodactylus* and *Epibdella*, which may be considered as characteristic members of their respective suborders, seems to offer very good embryological evidence that the separation of *Gyrodactylus* and its close relatives from the other members of the suborder Monopisthocotylinea was well warranted. Thus it seems as if the differences between the two groups are very fundamental and that they may be divided on the basis of their embryology as well as on the basis of their adult morphology.



TEXT FIG. 2. Diagrams showing the possible development of the intra-ovarian seminal receptacle. A. Relationships of seminal receptacle (*sr*), oviduct (*od*), and ovary (*ov*) in some of the other species of Monogena. B. A hypothetical intermediate stage. C. The relationship as found in *Epibdella melleni*. The boundary between the seminal receptacle and the ovary was not seen as a double wall, but it is quite probable that the two walls fused during development to form one wall as could be seen in sections.

The presence of the seminal receptacle within the ovary has not, to the knowledge of the authors, been reported previously in the order Trematoda. Inasmuch as a seminal receptacle is found as an evagination of the oviduct in other species of the Monogena, it is thought that the unusual seminal receptacle noted in *Epibdella melleni* is probably homologous with this structure in other species. Since the oviduct may be traced not only to but also inside of the ovary, it is thought that the intra-ovarian position of the seminal receptacle is brought about either by an overgrowth of the ovary around the seminal receptacle or by the invagination of the receptacle and basal portion of the oviduct within the ovary. Text figure 1 shows how such a rearrangement might have taken place. The wall around the receptacle could not be seen as a double wall as shown in the diagram, but it seems quite probable that the wall of the ovary and the wall of the receptacle might become fused during development, thus giving rise to one relatively thick wall as was seen in sections.

In view of the discrepancies between the original description of *Epibdella melleni* by MacCallum (1927) and the observations of the present authors, it is thought advisable that the description of the species be modified to read as follows:

Family Tristomidæ Taschenberg,
Subfamily Tristominæ Monticelli,
Genus *Epibdella* Blainville 1828,
Epibdella melleni MacCallum 1927.

Ectoparasitic on the eyes and epidermis and sometimes in the gill and nasal cavities of numerous marine fishes of the order Acanthopteri. Body oval, measuring 2–5 × 1.5–3.0 mm., with definite anterior suckers; skin smooth, posterior sucker with two pairs of hooks, one pair of spines, and fourteen very small (10 μ) larval hooklets, but no papillæ. Digestive tract greatly ramified. Cirrus sac pyriform, divided internally to form two saccules, the seminal vesicle and the prostatic reservoir. Two prostate glands; no vagina, uterus, nor genito-intestinal canal. Seminal receptacle within the ovary. Eggs tetrahedral, with two short hooks and one long filament.

Larva approximately 225 microns in length with three ciliated bands in the anterior, middle, and posterior regions of the body. Four large conspicuous eyes. Digestive system simple. Posterior sucker remains closed until after attachment. Development into adult is direct.

SUMMARY

1. The description of the adult of *Epibdella melleni* MacCallum 1927 is corrected and extended, and the complete life history of the parasite is described.

2. The morphology of the larva of *E. melleni*, including the excretory pattern, is described in detail.

3. The general outline of the development of the larva into the adult is traced.

4. Susceptibility to infection with *E. melleni* has been found to be limited to certain families of the order Acanthopteri.

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EXPLANATION OF PLATES

1. Camera lucida drawing of the adult of *Epibdella melleni* showing the principal organ systems. The vitellaria fill most of the interstitial space throughout the body and are not shown in the figure. Size of specimen is 4 millimeters.

2. Sagittal section through anterior half of adult specimen.

3. Sagittal section through the posterior sucker showing the secondary margin.

4. Free-swimming larva of *E. melleni*, drawn from living material, shortly after hatching. Size of specimen, 225 μ.

5. Larva which had been isolated when ciliated and maintained under a sealed coverslip for forty-five minutes, during which time the ciliated epithelium was shed and the posterior sucker opened.

6. Young individual obtained from an infected fish. Size of specimen, 320 μ.

7. Diagram of the excretory pattern of free-swimming larva.

8. Most common type of egg of *E. melleni.* Length of body of egg, 150 μ.

9. Somewhat less common type of egg.

10. Rather unusual form of egg which differs from the others in the ratio of the lengths of the edges of the tetrahedron.

11. Abnormal egg, shape probably the result of insufficient shell material in the oötype at time of formation.

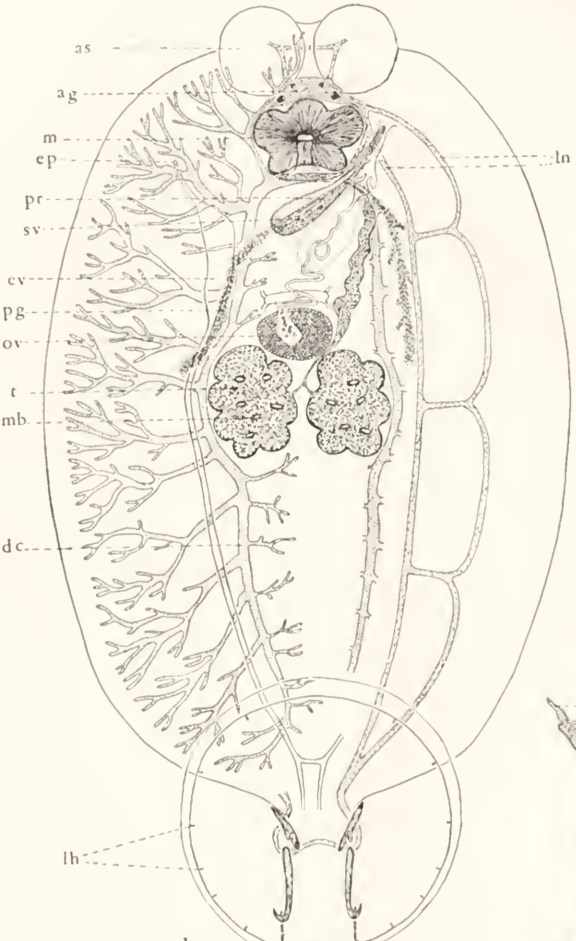
12. Filament as cast out when apparently still less shell material is available for formation of the body of the egg.

13. Larval hook. Specimen 10 μ in length. *a*, edge view; *b*, side view.

ABBREVIATIONS

ag	anterior nervous ganglion
as	anterior sucker
c	cirrus
cs	cirrus sac
dc	digestive cæcum
e	eye
ep	excretory pore
ev	excretory vesicle
gs	genital sinus
lh	larval hooks
ln	longitudinal nerve trunk
m	mouth
mb	muscular band through testis
mt	metraterm
od	oviduct
oes	œsophagus
ol	oral lips
ot	oötype
os	oral sucker
ov	ovary
pg	prostate gland
pog	post oral ganglion
pr	prostatic reservoir
sg	'shell gland'
sgd	'shell gland' duct
sm	secondary margin of posterior sucker
sr	seminal receptacle
sv	seminal vesicle
t	testis
vd	vas deferens
yd	vitelline duct
yr	yolk reservoir

PLATE 1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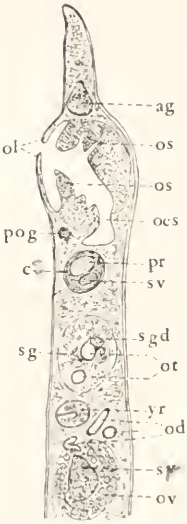
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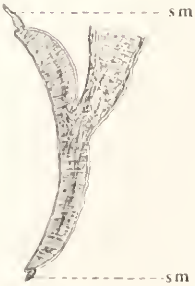
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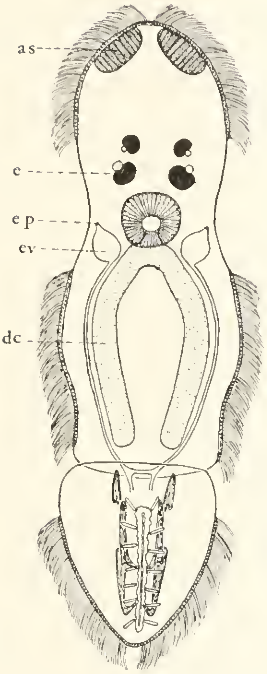
PLATE 2



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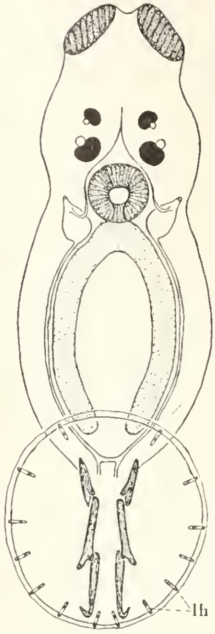

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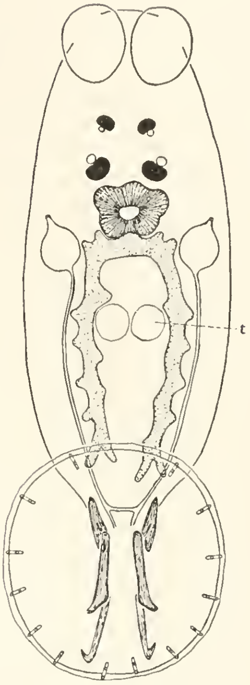

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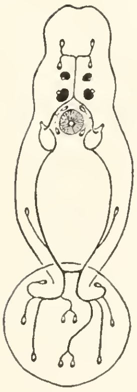

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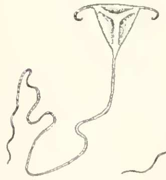

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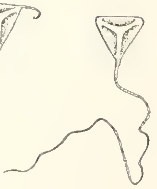

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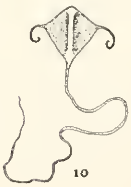

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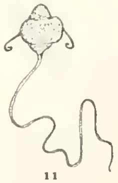

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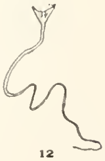

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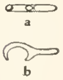

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THE VAPOR TENSION RELATIONS OF FROGS

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INTRODUCTION

An account of the water relations of frogs would be incomplete without a description of the exchanges of vapor between the animals and environing atmospheres. Some of the same properties of osmotic pressure and permeability that control water exchanges when frogs are surrounded by liquid might be expected to exhibit themselves in the presence of air. But whereas liquid water may serve as a medium for the exchange of other substances, water vapor is lost from the body without being accompanied by any dissolved material.

The two chief objects of the observations were: first, to find whether the rate of evaporation from the frog's body is proportional to the relative humidity of the atmosphere, or at least fixes a vapor tension curve for the organism; and second, to find whether a frog can come into equilibrium with a definite vapor tension. In theory every object has a measurable vapor tension; in practice the relation to water vapor is greatly complicated by the thermal properties of the body. It therefore becomes necessary to interpret data on rates of evaporation in terms of heat production, body temperature, and thermal conductivity.

Of practical consequence is the finding that under no circumstances can a frog absorb water from the atmosphere. Taken in connection with the fact that the skin offers no unusual obstruction to the loss of water into the atmosphere, it is evident that with respect to water balance a frog is unsuited to non-aquatic existence.

METHODS

In the course of the experiments six different procedures were used to establish the relationship between the frog and the atmosphere. Each method was instructive upon certain points and each was useful to evaluate some factors of water exchange. All the methods depended upon weighing the frog at frequent intervals; in these intervals only water was lost in appreciable amounts. The loss of carbon by a frog of 30 grams weight amounts to about 1.3 milligrams per hour, calculating from the mean rate of carbon dioxide production, measured by Smith (1925) on *Rana pipiens.* The same species was used in all the present measurements of evaporation.

The six methods of weighing evaporation losses that were used will be designated by letters. The last one is to be recommended for most general use in the study of atmospheric relations of organisms.

A. Frogs were exposed to the air of the room in a wire basket resting on a table. The temperature, relative humidity, and dew-point of the air were read at intervals. With inappreciable air motion and small changes of humidity during any one test, the weight changes could be related to the average humidity for the period.

B. A single frog was enclosed in a 400 cc. glass chamber through which conditioned air was recirculated. The chamber was suspended from a triple-beam balance, and connected by flexible rubber tubes with wash-bottles containing sulphuric acid mixtures. The air was pumped by raising and lowering a mercury bulb by means of a "wind-shield wiper"; the wash-bottles, in the bottoms of which were layers of mercury, serving as the valves of the pump. The apparatus was run in a room of constant air temperature, but this did not prevent water from condensing in the chamber when high humidities were used.

C. A single frog was placed in a screen cage in a 4-liter jar equipped with a fan. The fan was spun very rapidly by a belt and motor outside the jar, the fan shaft piercing a brass top fitted by a groove to the jar. The fan was stopped and the jar was opened each time the frog was to be weighed. In the bottom of the jar was a sulphuric acid mixture which controlled the relative humidity of the air. The fan and the air movement produced considerable heat so that temperature gradients always existed. The steady state was temporarily destroyed at each removal of the frog for weighing. The apparatus was operated in the constant temperature room.

D. The fan chamber was used, but the cage containing the frog was suspended from the balance arm by three wires passing through three holes in the chamber lid. The holes were closed by felt washers except when weighings were being made, at which time the fan was also stopped. The chamber was immersed in a regulated water-bath up to the rim, but most of the heat gradients persisted.

E. The frog rested on a screen platform in an ordinary desiccator above a sulphuric acid mixture. The desiccator was immersed in the water-bath up to the rim and was opened to remove the frog at each weighing. In weighing, the frog was exposed to another atmosphere and to handling, and the results were therefore unreliable at slow rates of drying. The air was quiet except as it was moved by the frog's breathing, but the frog remained at a constant distance (5 cm.) from the equilibrating solution.

F. A frog was suspended in a jar by a single wire from a balance arm. The jar and top were completely immersed in a water bath. The wire passed through a vertical tube in entering the jar, and the tube was ordinarily closed by a washer on the wire; when a weighing was taken the wire was raised slightly. The weights were reproducible enough so that an analytical balance was used. The cage proved to be a complicating factor because water coated its wires through capillary attraction away from the frog's skin. The most constant results were obtained by pithing the frog and suspending it by fine wires in a horizontal position. This method was accurately checked by exposing solutions of diverse vapor tensions in a glass dish in place of the frog.

High humidities were obtained by exposing the air to water or salt solutions of known concentrations. Lower humidities were controlled by keeping the air in contact with mixtures of sulphuric acid and water; their concentrations were estimated from their specific gravities measured with a Westphal balance. The vapor tensions of all these solutions were obtained from chemical handbooks.

Recovery from desiccation was studied in about half of the experiments. The frogs were placed in tap water and weighed at frequent intervals in the manner usual for wet frogs (Adolph, 1931).

RATES OF EVAPORATION

Frogs were gently blotted with a towel before each experiment so that no water would drip from their surfaces. Urine was pressed out of the bladder in the course of handling them, and it is well known that urine formation ceases when water is no longer being taken into the body (Adolph, 1927). Under these circumstances regular changes of body weight were observed. Temperature adjustments all occurred within the first half-hour; then weight was lost rapidly for one or two hours, after which the rate of loss was quite constant from hour to hour. This series of events is shown for one experiment in Fig. 1. Within the next 24 hours the rate of weight loss in any humidity usually decreased very slowly. This change of evaporation rate was

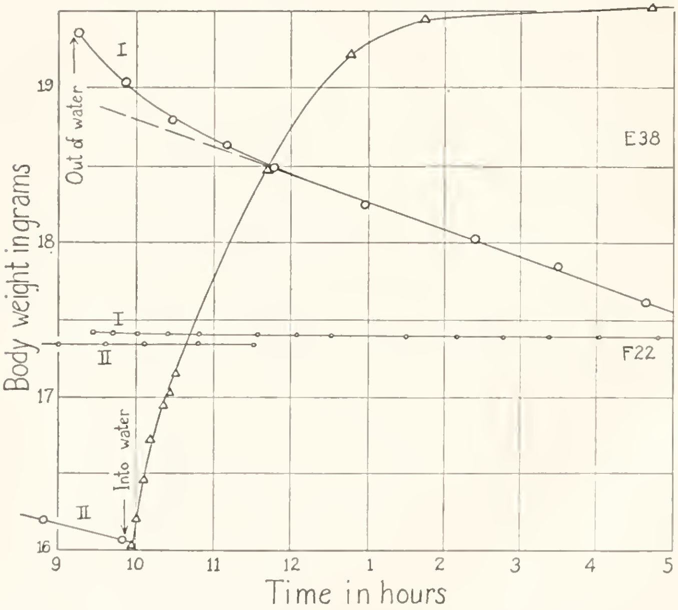

FIG. 1. Changes of body weight during evaporation (by method *E*) and recovery in tap water. The evaporation occurred in an atmosphere saturated with moisture at 20° C. during 25 hours, with slight decrease in rate throughout. For comparison of methods, the rate of evaporation by method *F*, during 26 hours exposure to a saturated atmosphere at 20° C., is also shown. In each case *I* and *II* represent successive days, each experiment being continued through the intervening night.

as great as 50 per cent when the atmosphere was saturated, but was less than 20 per cent when the humidity was nearly zero.

The responses of a single individual upon successive exposure to diverse humidities is shown in Fig. 2.

The various experiments were compared by plotting the rates of evaporation against the relative humidities that prevailed. It may be stated that no object is known for whose vapor equilibrium the absolute humidity has significance apart from the relative humidity. This principle follows from the kinetic behavior of gases at uniform temperature. In the experiments it was assumed that the relative humidity was that which would have prevailed if the atmosphere were completely in equilibrium with the equilibrating liquid; this liquid always exposed more surface than the frog. In many experiments a hair-hygrometer was placed in the chamber with the frog and this assumption was found to be nearly true.

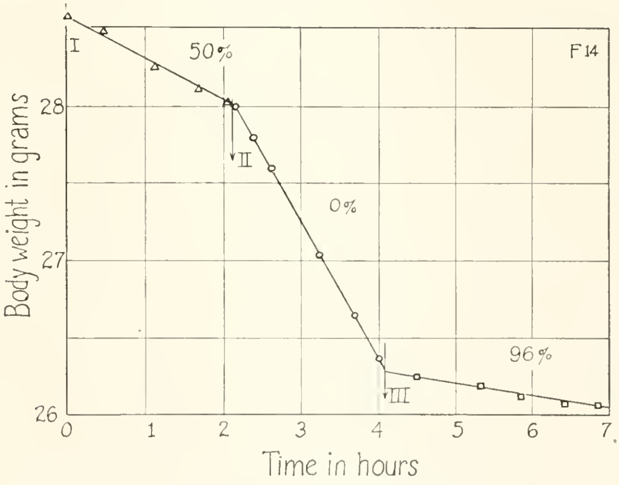

Fig. 2. Changes of body weight during successive exposures (by method *F*) to three relative humidities.

The rates of evaporation may be estimated in a number of ways; by finding the percentage of the original body weight lost in 24 hours, or the average number of grams of water lost per hour in the first six hours, or the grams per square centimeter of body surface lost during the steady state of the third to seventh hours of an experiment. Actually the experimental results were analysed in these several ways, and the last one was adopted, both as giving the most consistent and reproducible data and as being the most rational.

All the results by the six methods are shown as averages in Fig. 3.

Since the rates of evaporation by three different methods fall on curved lines, it is probable that the rates are not exactly proportional to relative humidities. This may be due to some feature of the experiment such as the gradient of vapor near the frog's skin and is not necessarily to be ascribed to the supply of moisture on the surface of the body. One important factor is that, as Hall and Root (1930) observed, the body temperature is much lower than the air temperature as the humidity declines.

A serious attempt was made to relate the rates of evaporation in high vapor tensions to the rates of water exchange by the frog immersed in salt solutions of the corresponding tensions. It is well known (Durig, 1901; Adolph, 1925) that in a certain range of sodium chloride solutions that are hypotonic when compared with the body fluids of the frog, water is gained by the body faster than it is gained in tap water. Was it possible that the vapor tension of the frog was higher at 99.8 per cent relative humidity than at 100 per cent? When

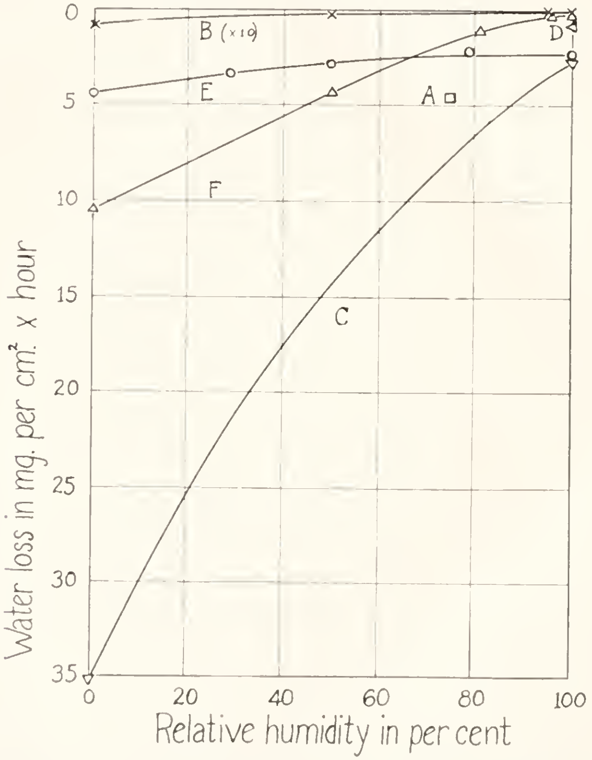

FIG. 3. Mean rates of evaporation at various relative humidities as obtained by the six methods.

compared (by method *C*), no significant difference in rate of evaporation was found at these two humidities. But this merely meant that the precision of the method was too low. In fact none of the methods was nearly good enough to decide this point, because the evaporation was slow even with high velocities of air motion, and because the production of heat by the frog as well as by the air motion could not be sufficiently corrected for.

Vapor Tension Equilibrium

Is it possible to establish conditions in which a frog will neither gain nor lose water? The only procedure sufficiently accurate to answer this question was method *F*. Some 30 experiments were run with saturated atmospheres; the air temperature being constant to ± 0.01° C., and fluctuations of weight due to all causes being reduced to ± 0.5 milligram per hour. The average result was a loss of 4.3 milligrams per hour by the frog, or perhaps 3.0 milligrams if the loss of carbon is allowed for. In 5 of the experiments slight gains of weight were shown and in 6 more experiments no change of weight occurred. But in every one of these 11 tests water was later found on the wire cage, and in all experiments where the wire cage was omitted and the pithed frog merely hung in the chamber some weight was lost. A few experiments where the humidity was reduced to 99.7 and 99.3 per cent showed similar losses of weight from the frog.

It is believed that no means could be devised of bringing a living frog into vapor tension equilibrium. The reason for this is, of course, that the organism is producing heat, and that at the frog's surface exists therefore a slight vapor deficit. A frog weighing 30 grams produces 12.5 calories per hour (Smith, 1925), and has a body surface of about 75 sq. cm. (Adolph, 1931); hence in a steady state it is losing 0.17 cal. per sq. cm. per hour. The evaporation from the frog of 4.3 milligrams of water per hour is equivalent to an expenditure of latent heat of 0.034 cal. per sq. cm. per hour. In other words, even this rate of evaporation dissipates only one-fifth of the heat that is being basally produced. Moreover, it eliminates between two and three times the amount of water that is being basally produced in the frog's body by oxidation, which is 1.6 milligrams per hour. The loss of 20 per cent of the frog's metabolic heat by evaporation in this atmosphere happens to be similar to the loss of 24 per cent of a man's metabolic heat by evaporation under basal conditions. It is obvious that no vapor equilibrium can be approached more closely than this by the metabolizing organism.

EFFECTS OF PITHING AND OF REMOVAL OF SKIN

The best determinations of evaporation rate, as already stated, could be made when the frog was totally quiescent and when the frog could be suspended by fine wires instead of being put into a cage. A few experiments were therefore made to compare the pithed frog with the normal frog. This was best done at high rates of evaporation, because of the smaller importance of absolute errors under such conditions. One experiment is shown in Fig. 4, and it is evident, as was true in other similar experiments, that no consistent difference existed in rates of evaporation between the pithed and the normal frog. A similar conclusion was reported by Hug (1927).

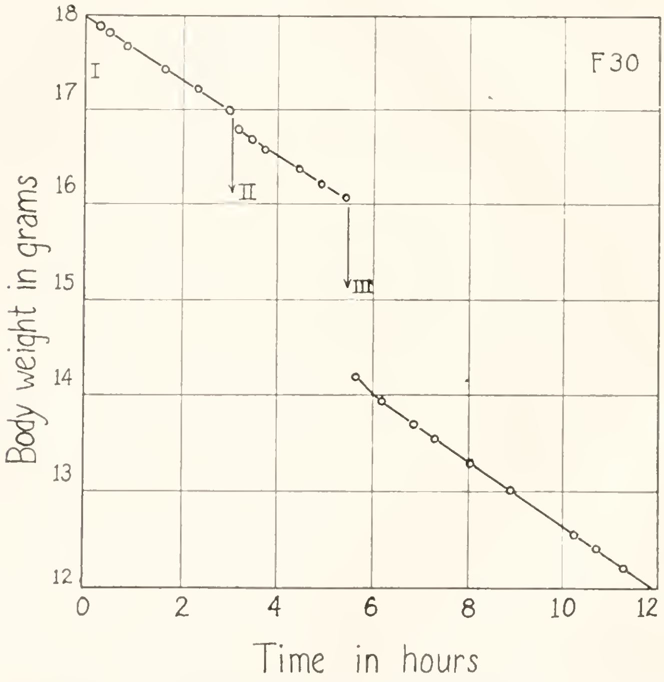

FIG. 4. Changes of body weight during successive exposures of the same frog under three conditions to a relative humidity of 50 per cent. *I*, normal; *II*, pithed; *III*, skinless. Method *F*.

After each experiment it was ascertained that the circulation of the blood persisted in the pithed individual. Two tests in which the circulation was completely stopped showed no detectable difference from the normal frog, and it is likely that the circulation is not a limiting factor in the rate of evaporation. It was concluded by Hug (1927) that dead frogs evaporated at the same rates as living ones.

During and after the evaporation tests it was noted that the appearance and feel of dryness in the skin was highly variable. But it proved impossible to correlate this condition with the rate or the amount of desiccation suffered.

In a short series of experiments the entire skins were removed from pithed frogs. It seemed possible that the external surface of the skin, being in equilibrium with fresh water, would naturally have the vapor tension of pure water, while the deeper tissues would have vapor tensions similar to that of a Ringer's solution, which corresponds to 99.7 per cent relative humidity. It was found that in low humidities the rate of evaporation of a skinless frog was little different from the rate of a normal frog, as Fig. 4 shows. This is in marked contrast to the protection against evaporation furnished by the skins of reptiles (Gray, 1928).

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In saturated atmospheres also no difference of rates could be measured. Whereas normal frogs lost 4.3 milligrams per hour, six skinless pithed frogs lost on the average 3.6 milligrams per hour, which is a much better agreement than could be expected. Obviously the heat production of the frog is sufficient to prevent water from condensing on the superficial tissues even though its vapor tension be slightly lower than the tension of pure water.

In a number of experiments frogs were first desiccated by 15 to 35 per cent of their body weights and then placed in saturated atmospheres. In no case was there a significant gain of weight; on the average the rate of loss was the same as for a normal frog. Even when the desiccated frogs were pithed and skinned no gains of weight were found. Evidently the vapor tension of the body cannot by this means be lowered sufficiently to overcome the vaporization due to dissipation of metabolic heat.

Rate of Regain of Water after Drying

But when put into water, a desiccated frog regains fluid at a rapid rate. The course of this regain is shown for one experiment in Fig. 1. The rate is fairly uniform for the first hour or two hours, though some gradual diminution in rate occurs. After the original weight of the frog has been attained, the gain ceases quite sharply.

The average initial rate of gain (38 experiments) was 0.8 gram per hour or 11 milligrams per square centimeter of body surface per hour. This is more rapid than the fastest desiccation in still air can be accomplished. The rate of regain is not correlated with the amount of desiccation, provided at least 5 per cent of the body weight had been lost, nor with the velocity of the desiccation.

Partial contact of the body with moisture is sufficient to supply water for regain (Stirling, 1877; Durig, 1901). If the dried-out frog is merely placed on a damp towel, water will be imbibed through the skin at the average rate. So far as is known to investigators generally, frogs never ingest water through the mouth when immersed in it.

It is of interest that the rate of respiratory metabolism increases with moderate desiccation of the frog and decreases markedly with extreme desiccation (Caldwell, 1925).

HEAT EXCHANGES

It has been demonstrated that the exchanges of water between frog and atmosphere do not correspond to an ordinary vapor equilibrium. The explanation is found in the continual production of heat in the body. In an atmosphere saturated with moisture at exactly body temperature this heat cannot be lost by radiation, nor by conduction, nor by convection. Evaporation is also impossible. Hence heat accumulates in the body until the surface temperature rises above that of the surroundings. With each fraction of a degree rise in temperature of the body, more conduction, convection and radiation become possible. The higher temperature now makes possible also evaporation into the warmed layer of air adjacent to the skin.

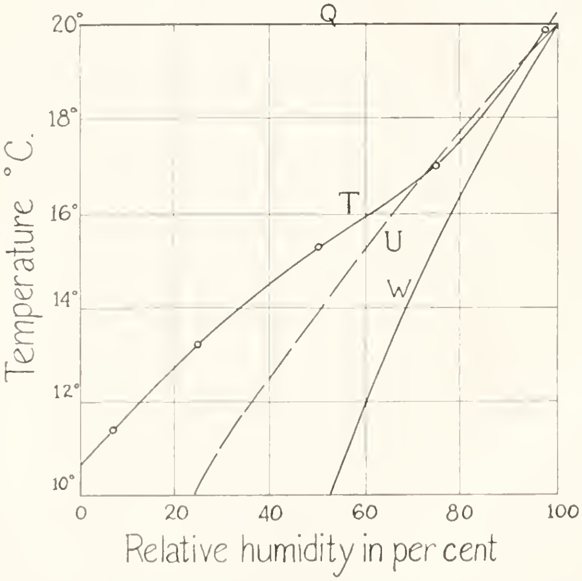

FIG. 5. Humidity and temperature relations at 20° C. *Q*, dry-bulb temperature of the air; *T*, rectal temperature of *Rana pipiens*, data taken from Hall and Root (1930); *U*, wet-bulb temperature of the air; *W*, dew-point temperature of the air.

The body temperatures of frogs in various relative humidities at 20° C. are supplied by the data of Hall and Root (1930); they are replotted in Fig. 5. The vertical distance between the lines *T* and *W* in this figure is the difference of temperature that exists in a steady state between a frog's body and the dew-point of the air surrounding it. This is least at 100 per cent humidity (0.25°), as might be expected.

Comparison with the wet-bulb temperature as obtained with a standard psychrometer (*U*), shows that a frog resembles a wet-bulb thermometer very closely in high humidities. In low humidities the effect of convection in slinging the psychrometer is more pronounced. Hall and Root (1930) had only slight air movement when they measured the rectal temperatures of the frogs.

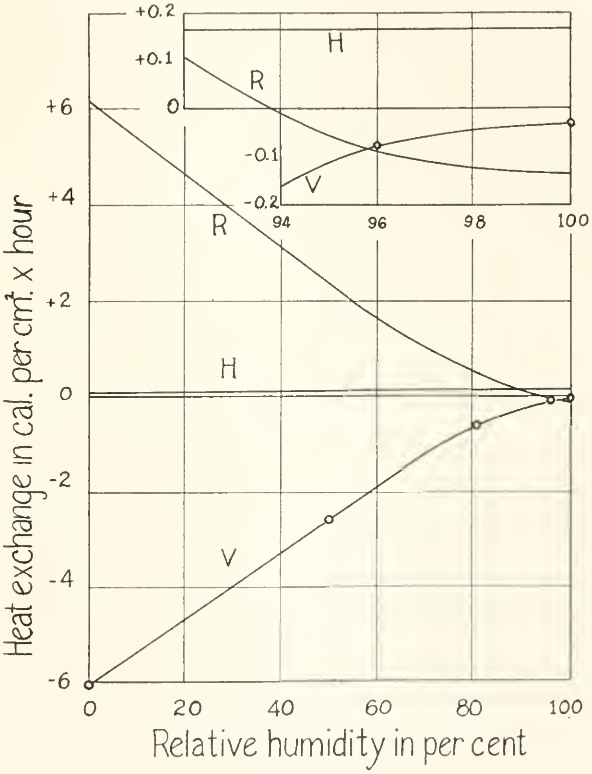

FIG. 6. Partition of virtual heat exchanges by frogs at 20° C. *R*, gain of heat by conduction, convection, and radiation from the surroundings; *H*, gain of heat by oxidative production in the frogs; *V*, loss of heat by evaporation of water from the frog's surface. The inset at the top is a ten-fold enlargement of the right-hand edge of the graph.

Evaporation as a complication of measurements of heat production has been discussed, at least for isolated tissues (Fischer, 1927; Hill, 1930). Heat production as a complication of measurements of evaporation deserves equal recognition.

From the rates of evaporation into air of diverse humidities it is now possible to estimate the proportions and total amounts of heat dissipated by vaporization on the one hand, and conduction, convection and radiation on the other hand. It is assumed for this purpose that all the water evaporated from the body gained its latent heat from the frog; this is very nearly true because the specific heat of the body is much higher than that of the air surrounding it. The view is equally sound that the frog really receives no heat from the surroundings while in the steady state, but merely acts as a converter of kinetic heat into latent heat within the atmosphere.

The calculations of heat production are facilitated by the data of Hall and Root (1930) on the body temperatures of frogs in various humidities (Fig. 5), and by the numerous data, as those of Vernon (1897) and Krogh (1914), on the relative rates of respiratory metabolism at various temperatures.

The partition of heat losses from the frog is indicated in Fig. 6, the rates of loss by evaporation being calculated from the measurements by method *F*. It will be seen that under nearly all atmospheric conditions evaporation alone removes heat much faster than combustion generates it. Ordinarily, therefore, the frog is virtually taking up heat from the surroundings by conduction, convection and radiation. It has been ascertained in the present experiments that in 100 per cent humidity, however, the evaporation accounts on the average for only one-fifth of the heat loss. Since the cooling is all produced by evaporation in proportion to the difference of vapor tensions between the frog's surface and the air, it is easily understood why there is no condensation of water on the surface of the frog even though it is much cooler than the atmosphere. The temperature of the frog never decreases to the dew-point of the atmosphere that surrounds it.

It is also possible to calculate roughly a coefficient of heat flow for the combined virtual losses by conduction, convection and radiation, excluding evaporation, from the data of Fig. 6. The heat dissipation (W) is proportional to the body surface (S) to the time (t), and to the temperature difference (θ). Or

$$W = kSt\theta.$$

The best value of k, for the range of low humidities where k is actually constant, calculated from the slopes of line *R* in Fig. 6 and line *T* in Fig. 5, is 1.0 cal. per square centimeter per hour per degree centigrade. It is more than possible that the curves present in the line for body temperatures (Fig. 5) and in the line for evaporation rates (Fig. 6) are significant, in which case *k* is modified at diverse high relative humidities, and the thermal properties of the frog's body differ at various humidities or body temperatures. Such differences might be due to vasomotor shifts or other physiological responses.

The amount of this heat flow that is due to the single factor of radiation can be calculated. It is assumed that the frog has maximal radiation (as for an ideal black body) such as is believed to hold true for human skin (Cobet, 1924), and that 70 per cent of its surface is exposed to radiation. It is then found that this form of heat transfer might account for half of the combined heat flow (*R*) at low humidities and for all of it at humidities above 70 per cent. Further, it can be calculated from the constant for heat conduction through air that a still atmosphere would be unable to conduct much of the other half of the heat from the water of the bath to the suspended frog. Hence convection currents set up by the frog's breathing and by temperature differences near the body must be important in bringing heat to the animal.

Comment

The failure of frogs to absorb water from moist atmospheres means that these animals cannot survive long away from liquids. While the habits of frogs are such as usually to keep them in or near water, toads are ordinarily regarded as terrestrial. Toads, when subjected to similar vapor tensions, likewise showed no ability to absorb vapor from a saturated atmosphere. Their survival away from water evidently depends upon their taking up water while in contact with wet objects; it has been seen that mere moisture held in towels can supply this. Soil is a sufficient natural source of supply. So far as is now known, a toad has no properties fitting it for water conservation or accretion that are not possessed by most aquatic animals.

The amounts of desiccation endured by frogs have always been matters for remark ever since the first observations were made by Edwards (1824), Chossat (1843) and Kunde (1857). Various investigators have attempted to find how much loss of water is consistent with subsequent recovery; loss of roughly 40 per cent of the body weight, which is 50 per cent of absolute water content, allows of survival (Snyder, 1908; Hall, 1922; Smith and Jackson, 1931).

Studies have been made of the relative losses by the various organs and tissues of the frog's body during desiccation (Durig, 1901;

Ueki, 1924; Iizuka, 1926; Smith and Jackson, 1931). At present little relation can be deciphered between the partition of water losses and the water economy of the body as a whole. It is possible that the marked loss of water by the skin helps to diminish the rate of subsequent evaporation to the small extent found above.

Summary

1. Frogs lose water by evaporation at rates that are nearly inversely proportional to the relative humidities of atmospheres.

2. Functioning of the central nervous system, of the blood's circulation, and of the skin made no significant differences in rates of evaporation.

3. In saturated atmospheres evaporation still goes on, which is explained by the fact that the production of heat keeps the body slightly warmer than the atmosphere.

4. In unsaturated atmospheres heat may be regarded as being lost by evaporation until the lowered temperature of the body comes into a steady state with the gain of heat by conduction, convection, and radiation from the surroundings.

5. No equilibrium of zero evaporation can be established for the living frog, and so the vapor tension of the frog's surface cannot be measured.

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A COMPARISON OF THE PLASTID WITH THE GOLGI ZONE

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There has always been a desire on the part of cytologists to homologize the structural elements of plant and animal cytoplasm. Of late much attention has been given to finding an element in the plant cell which might be comparable with the Golgi zone of the animal cell. Guilliermond (1929), because of the network structure assumed under certain conditions by the plant vacuoles, believed that these might be compared with the Golgi body. This idea found support in the work of Parat (1928), which seemed to show that the Golgi apparatus was mainly vacuolar in nature. Hall (1931), in a recent work on *Euglena*, maintains that it is not the sap vacuole which is comparable to the Golgi body but certain small granules staining with neutral red. Bowen (1920, 1922*a*, *b*, *c*), in an extensive study of insect spermatogenesis, has given a very definite criterion for recognizing the Golgi zone. It is that differentiated mass of cytoplasm which during spermatogenesis gives rise to the acrosome. Working with this in mind, he (1927) undertook a study of spermatogenesis in the moss, *Polytrichum juniperinum*. Unfortunately this investigation was never completed, but Bowen's belief was that the limosphere, a body which because of its development as described by Allen (1917) was thought by Wilson (1925) to be comparable to the acroblast, was elaborated by the osmiophilic platelets. Bowen's results, though differing slightly from the known formation of the acrosome in insects, made it seem possible that the osmiophilic platelets might be compared with the Golgi body.

The problem was attacked independently and in the same manner by Weier (1931, 1932) who, however, arrived at conclusions quite different from those of Bowen. During sporogenesis and spermatogenesis in *Polytrichum commune* reactions of the plastid to osmium and silver techniques and a strikingly comparable structure and behavior of the plastid to that presented by the Golgi body during comparable periods of development led this author to believe that the plastid and the Golgi zone had certain characteristics in common.

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This idea was not new, Bowen having suggested it in 1926 to withdraw it later in favor of the osmiophilic platelet hypothesis.

A study of spermatogenesis in *Polytrichum commune* and *Catharinaea undulata* furnished clear evidence that the limosphere is a derivative of the plastid. Indeed the history of plastid development during spermatogenesis is, in all important details, strikingly similar to the history of the development of the Golgi body during spermatogenesis in insects. When one adds to this the similarity in structure of the living Golgi zone (Morelle, 1926) with the living plastid (Zirkle, 1926), the similarity in reactions of both structures to certain fixing fluids (Morelle, 1926 and Weier, 1932), the similarity of function (the elaboration of specialized products for use by the organism), the similarity of relation to mitochondria in certain cells (Pensa, 1925; Parat, 1928), and the similarity in relation to chemically allied pigments (chlorophyll and hemoglobin—see Weier, 1932), it seems that there must be some significant relation existing between the plastid and the Golgi zone. Furthermore, it has been shown (Weier, 1932) that the previously supposed homology of mitochondria with plastid and of Golgi zone with vacuoles was not sound.

It is well known that osmium and silver impregnation techniques give preparations with a great variety of fixation pictures. Parat in his "vacuome" hypothesis has variously interpreted these to be "vacuoles," "active mitochondria," and "diffuse lipoids." During the course of the sporogenesis and spermatogenesis studies on *Polytrichum commune* and *Catharinaea* fixation pictures surprisingly similar to those interpreted by Parat as "vacuole" and "active mitochondria" were encountered in the plastid. This paper is a record of these fixation pictures. It is believed that the similarity found between Parat's "zone de Golgi" and the plastid is significant in two ways. Firstly, it further establishes the theory that the plastid is comparable to the region of cytoplasm which the zoölogists have generally called the Golgi apparatus. Secondly, it lends strong support to the interpretation of the Golgi body expressed in the work of Bowen, Nassonov, Gatenby, Hirschler, Beams, Morelle and others, namely, that the Golgi body or zone is composed of specialized living cytoplasm and not of a liquid vacuolar sap of a non-living nature as Parat holds.

Figure 1, *A* and *B*, is redrawn from a portion of Parat's (1928) Fig. IX, *a* and *b*. Both *A* and *B* represent, according to Parat, different fixation pictures of the "système vacuolaire" of the salivary gland of the *Chironomus* larva. In *A* fixation has been excellent; the "vacuoles" have impregnated evenly, presenting the aspect which one observes them to have in the living condition. In *B*, however, "l'élément apparaît incontestablement rétracté. . . . Les aspects les plus divers des vésicules chromophobes à parois chromophiles sont observables. . . ."

The plastid in the living cell of *Polytrichum* is formed of specialized cytoplasm sharply delimited from the remainder of the cell and, if it is a chloroplast, in some manner impregnated with chlorophyll. This specialized mass of cytoplasm appears quite homogeneous. Small starch grains may be observed within it after staining with iodine. Osmic acid impregnation upon occasion fixes and stains the plastid so that it appears perfectly homogeneous (Fig. 1, *C*). On the other hand, osmium methods, as well as other techniques, usually do not fix the plastid so perfectly. It most frequently appears in rather

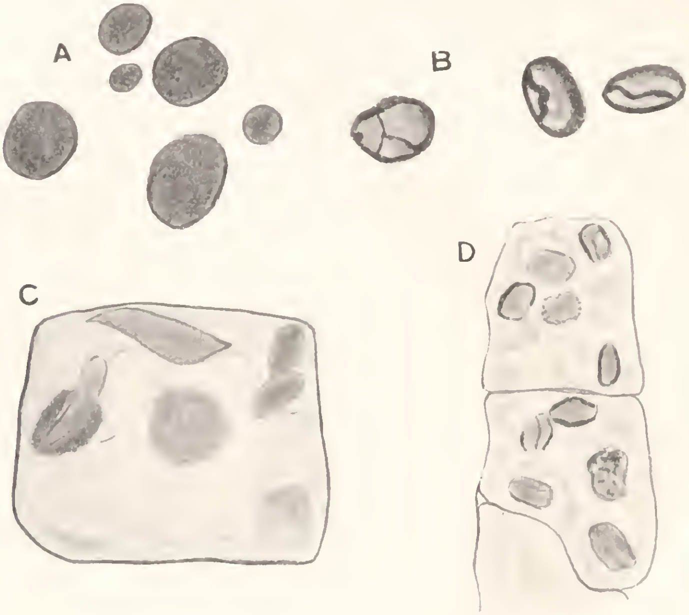

Fig. 1.

A and *B*. Two fragments of the salivary gland of the *Chironomus* larva after fixation according to Da Fano.

A. Cytoplasm insufficiently fixed: the impregnated vacuoles resemble dictyosomes. After Parat.

B. Cytoplasm well fixed: the impregnated vacuoles appear as they do in the living cell. After Parat.

C and *D*. Two aspects of plastid fixation in *Polytrichum commune*.

C. Cytoplasm well fixed: the plastid presents a homogeneous structure similar to that observed in living cells.

D. Cytoplasm poorly fixed: the structure of the plastid has changed so that it may appear similar to dictyosomes or it may have a simple plate-work transversing it.

diverse forms, commonly as a chromophobic mass of specialized cytoplasm with the border heavily stained, and a system of one or more plates running through the chromophobic mass, as in Fig. 1, *D*.

It seems then that the plastid and the "vacuole" both appear in the living state as more or less homogeneous masses. Fixation may upon occasion preserve this vital condition (Fig. 1, *A* and *C*). More frequently, however, fixation distorts plastid and "vacuole." Surprisingly enough the distortion is similar in both cases (Fig. 1, *B* and *D*), a "vesicule chromophobe à parois chromophile" or for the plastid a chromophobic mass of cytoplasm with a heavily staining border.

Parat reports a second illustration of poor and perfect fixation of the "vacuoles" in the oöcyte of *Helix*. In the living condition and after mitochondrial fixation the "vacuoles" appear as clear chromophobic regions in the ground cytoplasm of the cell. "Lépidosomes" together with some ordinary mitochondria gather around these areas. These two types of mitochondria are somewhat difficult to separate. In late stages of yolk formation the large "lépidosomes" come into a very intimate relationship with the "vacuoles." After silver impregnation the "vacuoles" contract, and in reducing the silver more heavily on one side come to appear strikingly like the dictyosomes of the classical Golgi body.

In the ovary of *Tulipa gessneriana* Pensa (1925, Figs. 19 and 20) describes an analogous behavior of the young plastids and mitochondria. After fixation according to Meves the young plastids appear as chromophobic regions similar to those which Parat, in *Helix*, has interpreted to be "vacuoles." Mitochondria are grouped around the plastids much as the "lépidosomes" of the *Helix* oöcyte gather around the "vacuoles." After silver impregnation the vacuole-like plastids of *Tulipa gessneriana* either form a small crescent-shaped network with only one side blackened by the silver or appear very much like the classical dictyosomes. That the regions of cytoplasm with which Pensa is working are plastids is perfectly clear from the presence within them of chlorophyll and starch.

Perhaps one of the most striking comparisons between the Golgi zone and the plastid is exhibited in Fig. 2, *A* and *B*. The Golgi zone in *A* is from Marg. and M. Parat (1930, Fig. III, *a*). It illustrates the appearance of the Golgi zone in the pelvic gland of *Triton marbre* after four hours preliminary fixation in the solution of Helly, followed by seventy-two hours post-chromatization at 38° C. This treatment mordants the Golgi zone so that it appears as a darkly-staining mass somewhat hollowed out by "vacuoles" in which the secretory grains first appear.

The plastid of *Zea mays* after identical treatment shows a very similar structure (Fig. 2, *B*). The specialized cytoplasmic mass is easily stained and is found to be furnished with "cavities." Under polarized light the black cross of starch showing within these "cavities" indicates that like the "vacuoles" of the Golgi zone they serve as depositories for the secretory products of the specialized mass of cytoplasm.

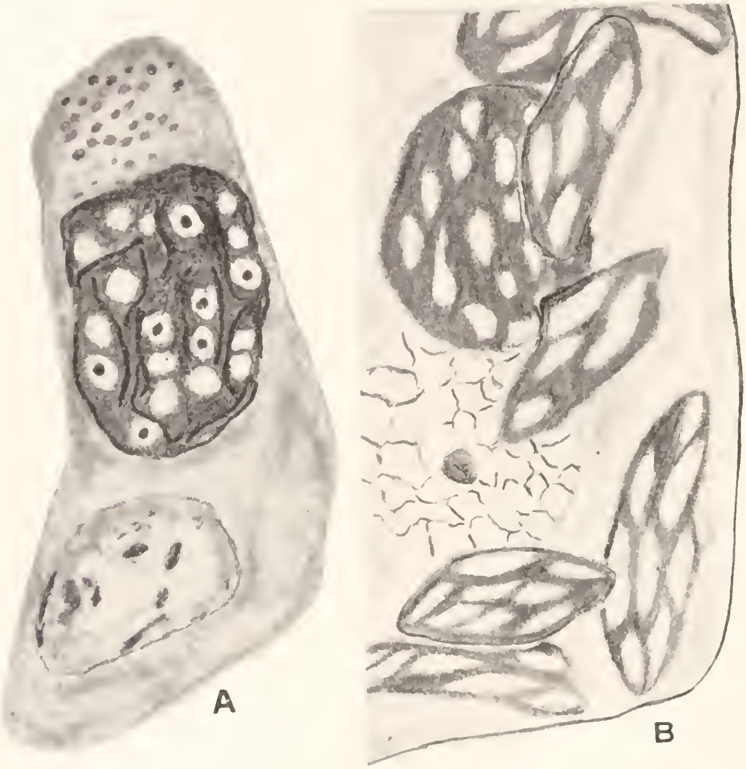

Fig. 2.

A. A cell from the pelvic gland of *Triton marbre* after a post-chromatization of 72 hours. The Golgi zone is a darkly-staining mass containing vacuoles, in which the secretion appears. Active mitochondria are also present. After Marg. and M. Parat.

B. A section of a cell from a *Zea mays* leaf after a post-chromatization of 72 hours. The plastid appears as a densely staining mass containing "vacuoles" in which polarized light demonstrates the presence of starch.

There is a difference, however, between Parat's description of the Golgi zone and the plastid: the presence of "active mitochondria" in the Golgi zone. According to Parat, a post-chromatization of six hours is not sufficient mordanting to make possible differential staining of the "active mitochondria." They do, however, show after treatment with osmium and silver. Parat describes these "active mitochondria" as being long filaments. Since they appear only after prolonged fixation and in both longitudinal and oblique sections (Figs. III *a*, IV and V, Marg. and M. Parat, 1930) or in longitudinal and cross sections (Figs. X, XVII *b*, *c*, *f*, *g*, Parat, 1928) as filaments and seldom as granules, is it not possible that these "active mitochondria" are in reality distinct plates such as Morelle and Bowen have described in the Golgi zone?

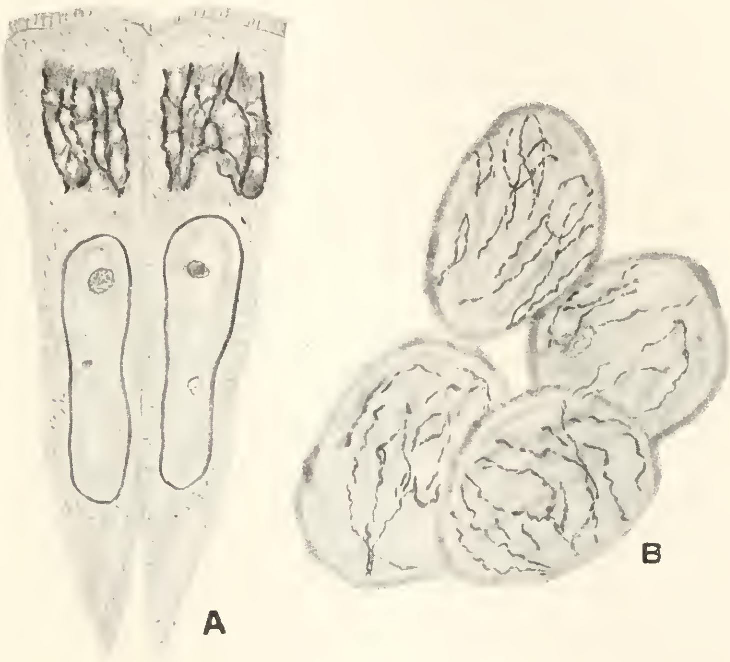

FIG. 3.

A. Silver impregnation of an intestinal cell of *Triton marbre*. Active mitochondria ramify through the diffuse lipoids of the Golgi zone. The vacuoles are frequently in contact with the active mitochondria. After Marg. and M. Parat.

B. Plastid of *Polytrichum commune* after osmium impregnation. Structures similar to the active mitochondria ramify through a denser region of cytoplasm and frequently border vacuole-like regions.

The plastid in *Zea mays* is evidently more resistant to the action of fixing fluids, for even after seventy-two hours post-chromatization it does not show any evidence of the platework structure which fixation usually imparts to the plastid as well as to the Golgi zone.

Figure 3, *A* and *B*, furnishes another striking comparison between plastid and Golgi zone. In this case the example of Golgi zone structure is taken from Plate XIV, *d* of Marg. and M. Parat, 1930. It represents a silver impregnation of an intestinal cell of *Triton marbre.* Figure 3, *B*, is an osmium impregnation of moss plastids.

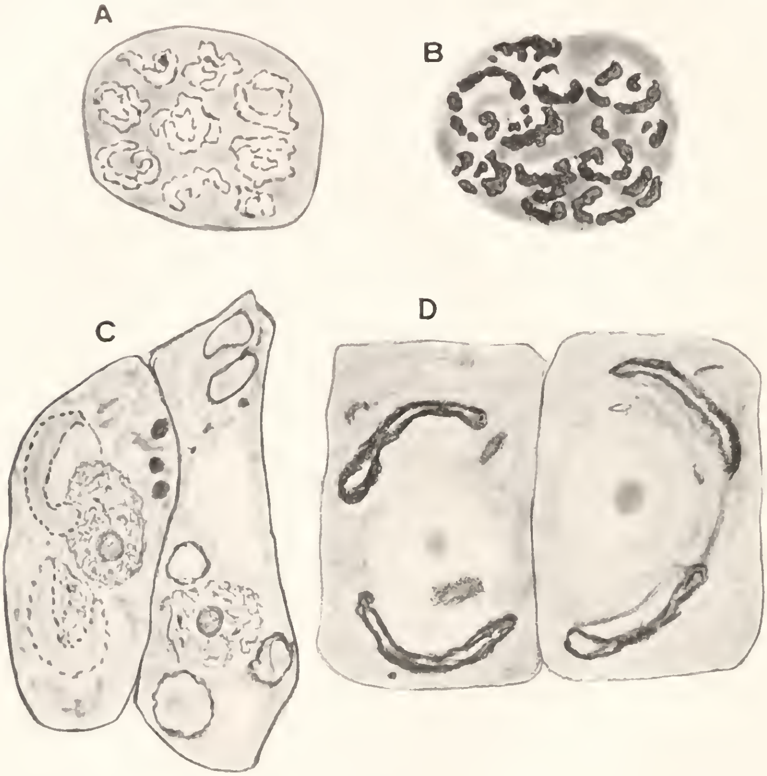

Fig. 4.

A and *B*. Epididymis cells of the mouse; osmium impregnation.

A. Transverse section. Perivacuolar chondriome impregnated. After Parat.

B. Heavy blackened ribbons, formed by the alteration of the perivacuolar chondriome, surround the vacuole, which is deformed but not impregnated. After Parat.

C and *D*. Types of osmic acid impregnation similar to *A* and *B* may be found to occur in the plastid of *Polytrichum commune.*

It will be noticed that in each case the "active mitochondria" lie quite parallel to each other and are most certainly in the case of the plastid and in all probability in *Triton marbre* delicate irregular plates. These ramify through a region of "diffuse lipoids" or specialized cytoplasm which has some power of reducing the osmium, thus appearing faintly gray. The "vacuoles" of the Golgi zone are clear and round, while the "cavities" of the plastid are longer and not so sharply and clearly delimited from the darker cytoplasm.

In Figs. 2 and 3 we have considered what apparently are good fixations of the "active chondriome" and "vacuome." However, after certain techniques, particularly osmium impregnation, this "active chondriome" may be greatly altered. Figure 4, *A* (Parat, 1928, Fig. XXVIII, *a*) is a cross section of an epididymis cell of the mouse. Here the "chondriome" is well preserved. By making a comparison with the longitudinal section shown in Fig. 3, *A* it may be seen that the "chondriosomes" are most frequently much more ribbon-shaped or plate-like than filamentous. They border on the "vacuoles" which have not blackened with the osmium. In Fig. 4, *B* (Parat, 1928, Fig. XXVIII, *b*) the "active chondriome" has been altered so as to form coarse black plates bordering the "vacuoles" which have not impregnated.

Under a similar treatment with osmium the archesporial plastid of *Polytrichum commune* shows a corresponding reaction. In Fig. 4, *C* an "active chondriome" or filamentous strands of what appears to be plastid cytoplasm border upon a non-blackened portion of the plastid. In Fig. 4, *D* heavy black plates are in contact with the region of the plastid cytoplasm which reduced no osmium.

Both plastid and Golgi zone, then, may under certain conditions precipitate osmium in the form of more or less heavy filaments or plates. These filaments or plates border on a non-reducing mass of plastid or Golgi substance. A comparison of the living plastid with this fixation picture shows that the reduction of the osmium is mainly confined to the borders of the plastonema of the archesporial plastid and that the plastosome, which is certainly of cytoplasmic nature, does not reduce the osmium. It appears lighter than the surrounding cytoplasm, or as a "vacuole." This comparison shows further that the structure of the plastid is not faithfully preserved in that the reduction of the osmium occurs most frequently at the interfaces of the plastonema, the plastosome and the surrounding cytoplasm. This suggests that the blackened regions, instead of marking formed cytoplasmic bodies, may rather indicate interfaces between certain specialized cytoplasmic regions where great cellular activity is taking place. The difference in amount of osmium reduced would depend upon either the length of time the tissue was treated with osmic acid or upon the amount of metabolic activity going on within the cell at the time of fixation. Since it seems that in the plastid this type of impregnation is an artifact in the sense that it does not preserve the exact details of the physical structure of the living cell, may it be suggested that perhaps a similar phenomenon has taken place in the Golgi zone? In other words, the "active mitochondria" are not mitochondria, permanent cellular elements, but mark instead, regions of intense cellular activity. This activity, as would be expected, is taking place at interfaces between differentiated regions of the cytoplasm, either between the Golgi zone and the surrounding cytoplasm or between differentiated regions within the zone.

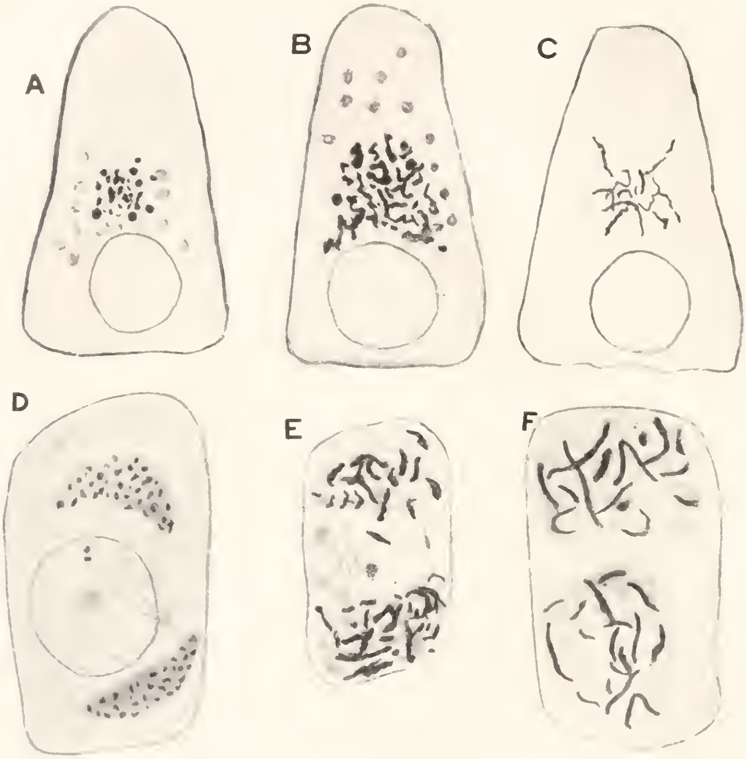

Fig. 5.

A, *B*, and *C*. Metallic impregnation of the pancreas cell of *Scyllium catulus*. *A* and *B*. The vacuome alone has impregnated. After Parat.

C. In this case only the active chondriome has impregnated. After Parat.

D, *E*, and *F*. Osmic acid impregnation of the androgonal plastid of *Polytrichum commune*. The same types of impregnation are encountered here as in *A*, *B*, and *C*.

It is, of course, known that mitochondria do invade the Golgi region (Morelle, 1926, Parat, 1928) but the "active mitochondria" are so different from the ordinary mitochondria in structure and reaction to fixation reagents and so similar to the artifacts due to fixation and osmification within the Golgi zone (Morelle) and within the plastid that one cannot avoid questioning the interpretation given to these structures by Parat.

In Fig. 5, *A*, *B* and *C* three different aspects of metallic impregnation of pancreatic cells have been reproduced from Parat's (1928) Fig. XXVI. In *A* and *B* only the "vacuome" has impregnated, while in *C* the "active mitochondria" alone reduced the osmium. The criterion for separating these two elements lies merely in the length and the thickness of the bodies which are able to be oxidized by the osmium. Corresponding types of osmium impregnation are frequently encountered in the plastids of androgones within the same antheridium (Fig. 5, *D*, *E* and *F*). In the case of the plastid, one is dealing with neither "vacuoles" nor "active mitochondria" but probably with especially active regions of plastid cytoplasm.

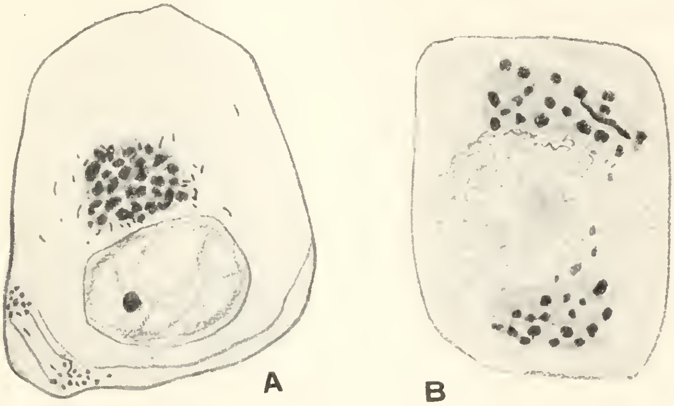

FIG. 6.

A. Nervous cell of the tadpole of *Bufo vulgaris*. Silver impregnation. Vacuome is impregnated as well as a portion of the active chondriome. In the satellite cell the vacuome is impregnated and exhibits a definite polarity. After Parat.

B. Androgonal cell of *Polytrichum commune*. Osmic acid impregnation. Vacuole-like regions impregnate within the plastid.

Even the Golgi bodies of nerve cells are not without a fixation picture which may be compared to the plastid. Silver treatment on this type of tissue in *Bufo vulgaris* results in an impregnation of the "vacuoles" and "active mitochondria" embedded in a mass of cytoplasm which is more dense than that of the remainder of the cell (Fig. 6, *A*, redrawn from Parat, 1928, Fig. XIV, *b*). At the lower left-hand corner of the large cell is a small satellite cell with its "vacuome" impregnated and showing a definite polarity strikingly similar to that shown by the plastids when there are but two per cell (Fig. 6, *B*).

The plastid of *Polytrichum commune* (Fig. 6, *B*) after treatment with osmic acid not infrequently presents an image quite similar to that assumed by the Golgi zone in the nerve cell of Fig. 6, *A*. Many small regions reduce the osmium intensively so that the plastid appears to have a very similar structure to the Golgi zone of *Bufo vulgaris*. However, the regions in the plastid which reduce the osmium so strongly are apparently not the "cavities" which previously have been compared to Parat's "vacuoles" and within which the starch first appears. They probably represent regions of cellular activity rather than any definite morphological cellular element. The two plastids possess a definite polarity similar to that shown by the "vacuoles" in the satellite cell of Fig. 6, *A*. May it not be that the "vacuoles" too are regions of active cytoplasm rather than cellular elements perpetuating themselves only by division as the nucleus does?

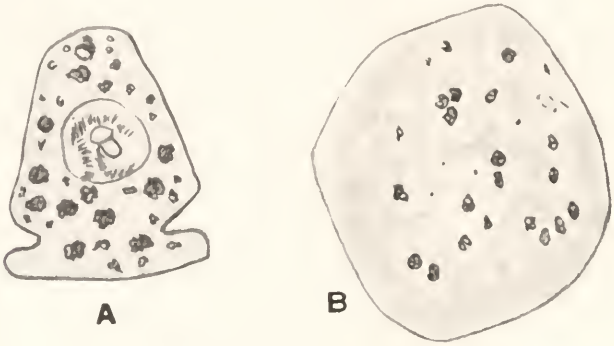

FIG. 7.

A. Salivary gland of the *Chironomus* larva. Vital. The vacuoles appear to be in the form of a network. After Parat.

B. Proplastids in microsporocyte of *Zea mays*. Benda. The proplastids are reticular.

Parat's classical example of vacuole formation is the salivary gland of the *Chironomus* larva (Parat, 1928, Fig. V, 1 to 8). In the account of this development there are present in all eight stages small discrete "vacuoles." At stage six, however, some of these flow together, forming the secretion. It seems that Parat may have confused the secretory products with the discrete Golgi elements, the region of cytoplasm which elaborated the products.

At any rate young plastids frequently may be observed to have network aspect very similar to Parat's "vacuoles" found in the salivary gland of the *Chironomus* larva. Pensa has reported young plastids of this same structure as occurring in the cells of the young ovary of *Tulipa gessneria*. In Fig. 7, *B* young plastids found in *Zea mays* microsporocytes are seen to compare very favorably with the "vacuoles" of the salivary gland of the *Chironomus* larva (Fig. 7, *A*).

During spermatogenesis the Golgi zone is formed, according to Parat, of "lépidosomes" or specialized mitochondria, "rhagiocrine and plasmocrine vacuoles," and "diffuse lipoids." The pro-acrosomic granules are associated with the "rhagiocrine vacuoles." Here the "lépidosomes," rather than the vacuoles, as in yolk formation, take on the form of the dictyosome. If we compare the dictyosome-like "lépidosome" (Fig. 8, *A*) with the archesporial and androgonal plastids (Fig. 8, *B*), we find no appreciable difference in structure; both are formed of a convex chromophilic border and an internal chromophobic substance.

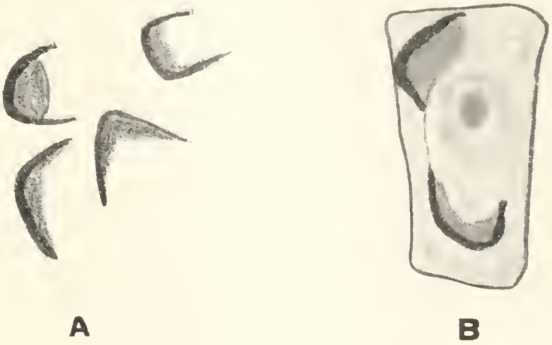

FIG. 8.

A. Idiosome from the spermatocyte of *Helix pomatia*. Osmic acid impregnation. The lépidosomes with convex chromophilic and internal chromophobic portions resemble the classical dictyosomes. After Parat.

B. Archesporial plastid from *Polytrichum commune*. Benda or osmium fixation. The plastid closely resembles the dictyosomes.

This might be taken as an indication that the plastid, too, is a specialized sort of chondriosome comparable to the "lépidosome." However, in view of the previous comparison between the plastid and the Golgi zone and the part played by the plastid in the elaboration of the limosphere, this is hardly possible. The rôle of the plastid in the latter respect is identical with that of the classical Golgi body, it elaborates the apical body or acrosome. Parat claims that the "lépidisomes" do not enter into the elaboration of the acrosome. He, however, stresses the fact that they are in an intimate relation with the Golgi zone up until the deposition of the acrosome. Exactly at this time they move away from the zone and pass down the tail of the maturing sperm. It seems that Parat may have missed one of the most important steps in spermatogenesis, the elaboration of the acrosome by the acroblast.

It does not seem possible that the "lépidosome," which is so strikingly similar in behavior and structure to the dictyosomes and androgonal plastid, does not play some rôle in the formation of the acrosome. Nor does it seem possible that the excellent preparations of the late Professor Bowen which the present author has had the privilege of examining can all be either special cases or all distorted to show a relationship between acroblast and acrosome which does not exist in life.

It seems that the similarity shown by the plastid to the Golgi zone cannot be without some significance. It must mean that these two regions of the cell are comparable, possibly even homologous, although the great evolutionary distance between the mosses and animal forms under consideration makes such a suggestion extremely hypothetical.

Since we know that the plastid is essentially cytoplasmic, that it possesses a few "cavities" (probably also cytoplasmic in nature) in which the starch is deposited, that it gives rise upon fixation to a peculiar platework picture, in all likelihood marking particularly active regions within the plastid, and that it gives rise to the apical body in spermatogenesis, we cannot but question Parat's interpretation of the plate-like "active mitochondria," the secretory "vacuoles" and the "diffuse lipoids" of the "zone de Golgi."

If the similarity shown by the plastid to the Golgi zone in structure, function and behavior may be used as evidence for the structure of the Golgi zone, it lends strong support to the interpretation placed upon that cellular element by Morelle, namely, that it is a mass of specialized cytoplasm containing a number of "vacuoles," and that after prolonged treatment with fixing agents a coarse, heavily-staining network appears within the specialized cytoplasmic mass.

I wish to acknowledge my indebtedness to Professor L. W. Sharp for the interest which he has shown in the progress of this work, and for his stimulating and helpful criticisms of the many perplexing questions which have arisen in connection with the study; and to Professor E. B. Wilson for his kindness in loaning me some of the preparations of the late Professor Robert Bowen.

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PHYSICAL AND CHEMICAL CONSTANTS OF THE EGG OF THE SEA URCHIN, ARBACIA PUNCTULATA

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For purposes of calculation it would frequently be convenient to have readily accessible data regarding the physical characteristics of much-used animal and plant cells. The unfertilized egg of the sea urchin, *Arbacia punctulata*, is a cell in a relatively stable condition, which has been studied perhaps more widely than any other marine material. Accordingly, I have collected the available data regarding this egg, with the hope that others will do a similar thing for different cells and organisms. A table of physiological constants for man should prove most valuable.

The figures for the unfertilized sea urchin egg are given in metric units for the temperature indicated (mostly "room" temperature, about 23° C.) with temperature coefficients where these are known. Although the cm.-gram-second units are common in physics, the micron-milligram-minute units give more convenient figures in dealing with single cells and have been more widely used by physiologists. It must always be remembered that there is considerable variation in the eggs of different females and less variation in the eggs of a single female (see Goldforb, 1917; for effects of ageing, Goldforb, 1918, *a* and *b*). The most probable average value is, therefore, taken as a basis for derived values. Some determinations cannot be made with great accuracy and consequently represent approximations, such as the diameter of granules and percentage of granules, and determinations based on such measurements. Where our knowledge of data is not complete, this compilation may serve as a guide to future investigation.

1. Dimensions

Egg diameter,[1] 74 μ, average of 550 cells from four females. Eggs do not flatten under their own weight (McCutcheon, Lucké and Hartline, 1931); 66 to 94 μ (Glaser, 1914). Loss of diameter on fertilization, 2.3 μ for 74 μ egg (Glaser, 1924).[2]

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[1] The European *Arbacia pustulosa* egg has a diameter of about 100 μ.

Egg surface, 17200 μ^2.

Egg volume, 212000 μ^3.

Diameter and volume of eggs separated by centrifugal force. Colorless and pigmented half separate from whole egg and clear and granular quarter from colorless half (E. B. Harvey, 1932*a*).

	Diameter μ	Volume μ^3	
Whole egg	73	203690	(197400 μ^3 Lucké, 1932)
Colorless half egg	60	113100	(108600 μ^3 Lucké, 1932)
Pigmented half egg	56	91950	(90700 μ^3 Lucké, 1932)
Clear quarter egg	53	77950	
Granular quarter egg	40	33510	

Thickness of jelly, 28 to 32 μ (E. B. Harvey).[3]

Thickness of perivitelline space, 3–5 μ (E. B. Harvey).

Thickness of hyaline plasma layer (ectoplasmic layer), 1–2 μ (E. B. Harvey).

Diameter nucleus, 11.5 μ (E. B. Harvey).

Diameter oil globules, 1 μ (E. B. Harvey).

Diameter fifth layer granules,[4] 0.6–1 μ (E. B. Harvey).

Diameter yolk granules,[5] 0.7–1.1 μ (E. B. Harvey); 0.3 μ (Heilbrunn, 1926).

Diameter pigment granules, 1.1–1.6 μ (E. B. Harvey); 0.92 μ (Heilbrunn, 1926).

Percentage formed bodies (E. N. Harvey).[6] Nucleus 0.4; oil globules 1.0; fifth layer 4.8; yolk 27.2; pigment 5.5; fluid 61.1.

Number of chromosomes (diploid), probably 38 (Morgan, 1927).

2. Densities

Sea water, 1.024 (Lyon, 1907). 1.02426 21.5° C./21.5° C. (Garrey, 1905).

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[2] This has been questioned by others.

[3] The undated references to E. B. Harvey apply to data furnished by her for this article but not published elsewhere.

[4] This layer appears on top of the yolk layer when eggs are centrifuged for a long time. The granules are very variable in size and shape. The figures for granule diameter are only approximate.

[5] These granules are often irregular and frequently polyhedral in centrifuged eggs.

[6] The percentage nucleus was calculated from its volume based on diameter measurements. The percentage of other formed bodies was made by measuring the dimensions of the spherical segment occupied by the layers in long centrifuged eggs, calculating the volume of the spherical segment and taking 74 per cent of this, since spheres of equal size lying in a volume occupy 74 per cent of that volume. The figures must be considered only approximate, as it is not certain how much packing of the granules occurs.

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Egg with jelly, 1.090 (E. N. Harvey, 1931).[7]
Egg without jelly, 1.084 (E. N. Harvey, 1931).[7] } 1.081–1.087 (Lyon, 1907).[8] 1.0485–1.0656 (Heilbrunn, 1926*b*).[9]

Density oil? [10]

Density nucleus?

Density clear layer, 1.0358–1.0514 (Heilbrunn, 1926*b*).[11]

Density fifth layer granules?

Density yolk granules, 1.1035–1.1269 (Heilbrunn, 1926*b*).[12]

Density pigment granules?

Density clear half of egg separated by centrifugal force, 1.076 (E. N. Harvey, 1931).[7]

Density yolk half of egg separated by centrifugal force, > 1.100 (E. N. Harvey, 1931).[7]

3. VISCOSITY

Water = 0.01 poise (dyne-seconds per cm.^2) at 20° C.

Granule-free protoplasm, 0.018–0.025 (approximately .02) poise[13] (Heilbrunn, 1926, *a* and *b*). Granule-free protoplasm, 0.04 (Heilbrunn, 1928).[14] (See this book for effect of substances on viscosity.)

Entire protoplasm, 2–3 times above.

Fertilized egg considerably more viscous. For changes during development see Heilbrunn, 1920.

For effect of CO_2 see Jacobs, 1922; for effect of fatty acid-salt buffers see Howard, 1931; for effect of acids and alkalies see Barth, 1929; for effect of temperature see Heilbrunn, 1924.

4. TENSION AT SURFACE

Air/water surface tension = 73 dynes per cm. at 20° C.

Unfertilized egg less than 0.2 dynes per cm., with considerable variation, for 25 per cent increase in surface area.[15] (E. N. Harvey, 1931.)

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[7] Determined by centrifuging eggs in mixtures of sea water and .95*m* cane sugar at 22° C. and measuring the density of the sea water-sugar mixture in which they remain suspended with a hydrometer graduated for 60° F./60° F. There is considerable variation in density of different eggs. *m* = molal, 342 grams cane sugar to a liter of water.

[8] Determined by centrifuging eggs in gum arabic in sea water. No difference in density up to 16-cell stage but pluteus has a density of 1.055 to 1.066.

[9] Determined by centrifuging eggs in cane sugar solutions whose densities were determined from tables.

[10] The relative density of oil, nucleus, etc., is in the order given, oil and nucleus being lighter than clear layer, the remaining granules heavier.

[11] Calculated from density of whole egg and volume (19 per cent) occupied by heavy granules of egg.

[12] Determined by crushing eggs and centrifuging crushed material in sugar solutions of various densities.

[13] Determined by centrifuging granules and applying Stokes' law with Cunningham correction.

[14] Determined by Brownian movement method.

Initial tension 0.08 dynes per cm. for undistorted egg (fertilized or unfertilized) and approximately proportional to deformation (flattening) and to increase in surface area.[16] Surface has properties of elastic membrane. Increase of surface force for 1 per cent increase of surface area is 0.005 dynes per cm. for unfertilized and 0.7 dynes per cm. for fertilized egg. Internal pressure about 40 dynes per cm.^{2} (Cole, 1932.)

5. Osmotic Properties

Osmotic pressure equals that of sea water (22 atmospheres at 0° C.), whose depression of freezing point, Δ, $= -1.81°$ C. (-1.805 to -1.84). NaCl 0.52M, $MgCl_2$ 0.29M and cane sugar 0.73M (342.2 grams per liter of solution) have same Δ as sea water. (Garrey, 1905, 1915.)

Equilibrium given by $(V_0 - b)P_0 = (V_{ex} - b)P_{ex}$, where V_0 = volume in sea water, P_0 = osmotic pressure sea water, V_{ex} = volume in concentrated or diluted sea water, P_{ex} = osmotic pressure in concentrated or diluted sea water, b = osmotically inactive material, 11 per cent (7 to 14 per cent) (McCutcheon, Lucké and Hartline, 1931). No effect of narcotics on equilibrium.

Permeability (k) **to water** defined as:

$$\frac{dV}{dt} = kA(P - P_{ex}),$$

where dV/dt = rate of change in volume, A = surface area, P = osmotic pressure at time t, P_{ex} = osmotic pressure within egg or of solution with which cell is in equilibrium. For water entering eggs at 20° C., $k = 0.087\ \mu^3$ per μ^2 surface per atmosphere difference of pressure per minute; for water leaving eggs, $k = 0.141\ \mu^3$. At 15° C., $k = 0.05$–0.06 for endosmosis and 0.07–0.08 for exosmosis. Values are independent of osmotic pressure but depend on kind and proportions of salt in medium, injury, narcotics, etc. $Q_{10} = 2$ to 3 between 12° and 24° C. Thermal $\mu = 15200$. (Lucké, Hartline and McCutcheon, 1931.) See also Northrop, 1927; McCutcheon and Lucké, 1926 and 1927.

For fertilized eggs $k = 0.12$ to 0.17 at 15° C. for endosmosis (Lucké, unpublished).

Lillie (1916; 1917) finds water enters fertilized eggs and butyric acid activated eggs four times more rapidly than unfertilized.

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[15] Determined by centrifugal force necessary to pull egg into two halves.

[16] Determined by force necessary to deform eggs by flattening.

Absence of ions (glucose solution) increases k from 0.05 to 0.1 at 12° C. and 0.0001M $CaCl_2$ or $MgCl_2$ added to glucose solution maintains k same as in sea water (McCutcheon and Lucké, 1928).

Cations decrease permeability to water, the effectiveness increasing with the valence of the cation. In 0.38*m* dextrose solution containing 0.005M K_3 citrate (in which solution cells have high water permeability), the following concentrations of cobaltammine chlorides were required to reduce permeability to the value obtained in sea water:—0.00005M of the 6 valent salt, more than twice as much of the 4 valent salt, more than eight times as much of the 3 valent, and 64 times as much of the 2 valent salt, while this amount of the 1 valent salt was incompletely effective. Temperature 12° ± 0.5° C. (Lucké and McCutcheon, 1929).

Anions increase permeability to water, the effectiveness increasing rapidly with the valence of the anions. In 0.38*m* dextrose solution containing 0.0005M $CaCl_2$, 0.001M of potassium ferrocyanide was required to definitely increase permeability, twice as much ferricyanide, four times as much potassium sulphate, and eight times as much chloride. Temperature 12° ± 0.5° C. (Lucké and McCutcheon, 1929).

Narcotics (urethanes and carbamates) in sea water do not decrease permeability to water beyond the value normally found in sea water. When dissolved in a non-electrolyte solution, they tend to decrease permeability to water. Thus, 0.025M *n*-butyl carbamate in 0.38*m* dextrose solution decreased permeability from 0.096 (the value in dextrose solution alone) to 0.062. Temperature 15° ± 0.5° C. (Lucké, 1931).

For effects of HCN and KCN on permeability to water see Blumenthal, 1927; for ether see Heilbrunn, 1925; for anaesthetics and KCN see also Lillie, R. S., 1918, and Blumenthal, 1928.

6. PERMEABILITY

Permeability to a solute (S) may be defined as

$$\frac{dS}{dt} = kA\left(C_s - \frac{S}{V}\right)$$

where dS/dt = rate of change of amount of solute; A = surface area; C_s = external concentration; V = volume of the egg. k = number of mols that will penetrate $1\mu^2$ of surface in 1 minute with a concentration difference between exterior and interior of 1 mol per liter and is[17] (Jacobs and Stewart, 1932):

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[17] Determined by change in volume of eggs in hypertonic solutions.

Ethylene glycol	3.6×10^{-15}
Acetamid	5.8×10^{-15}
Propionamid	14.2×10^{-15}
Butyramid	36.6×10^{-15}
Glycerol	0.5×10^{-15}

For fertilized eggs k for ethylene glycol $= 9.8 \times 10^{-15}$ (Stewart and Jacobs, 1932).

For permeability to non-electrolytes and NH_4 salts see Stewart, 1931; for fatty acid-salt buffers see Howard, 1931.

7. Electrical Properties

Electrical resistance.[18]—Interior 90 ohm-cm. or 3.6 times that of sea water at room temperature for 1000 to 15×10^6 cycles. Impedance of surface high below 1000 cycles and behaves like a "polarization-capacity." No measurable change in resistance on fertilization (Cole, 1928).

Dielectric constant?

Cataphoretic potential,[19] $-$ 0.035 volts with jelly; $-$ 0.021 volts without jelly for zeta potential (K. Dan, 1931).

8. Hydrogen-ion Concentration

pH of sea water, 8.2.

pH of egg? Probably 6.6–6.8 from studies of other echinoderms by indicator method. No change on fertilization. Change to 5.4–5.6 on injury. Nucleus of immature egg 7.6–7.8 (Chambers and Pollak, 1927; Needham, 1926).

Buffer value?

9. Metabolism

Heat production,[20] $-$ 0.08 gram-cal. per 10^6 eggs of 74 μ diameter per hour; 0.88 gram-cal. on fertilization, falling to 0.58 gram-cal. 20 minutes after fertilization and 0.52 gram-cal. per 10^6 eggs per hour at first cleavage (50 minutes after fertilization). 3.34 ergs per egg per hour for unfertilized and 20 ergs per egg per hour for fertilized eggs (Rogers and Cole, 1925).

Carbon-dioxide production? See Lyon, 1904.

Oxygen consumption, 33.6 mm.^{3} (17–51 mm.^{3}) measured at 24.7° C. O_2 per 10^6 eggs (diameter 72–80 μ; average 77 μ) per hour at 24.7° C. or 0.0023 mm.^{3} O_2 per mm.^{3} egg per minute. Fertilized eggs four times with no variation during cleavage[21] (Tang, 1931). 30 mm. per 10^6 eggs (diameter 72 μ) per hour at 25° C. .0025 mm.^{3} O_2 per mm.^{3}

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[18] Determined by vacuum thermocouple voltmeter-ammeter method.

[19] Determined by deflection from vertical fall in electric field.

[20] Determined by thermocouple and twin-calorimeters.

[21] Warburg manometer method.

egg per minute. Fertilized eggs 5 times greater with no variation during cleavage[21] (Tang and Gerard).

Temperature coefficient of oxygen consumption[22] (Loeb and Wasteneys, 1911*a*).

° C.	Q_{10}	° C.	Q_{10}	° C.	Q_{10}
3–13........	2.18	10–20......	2.17	17–27......	2.0
5–15........	2.16	13–23......	2.45	20–30......	1.96
7–17........	2.00	15–25......	2.24		

Oxygen consumption of unfertilized eggs at different O_2 tensions. 760 to 50 mm. O_2, 100 per cent; 45 mm., 95 per cent; 20 mm., 90 per cent; 10 mm., 70 per cent; 6 mm., 55 per cent; 2 mm., 40 per cent (Tang, 1931)[21] at 24.7° C.; see Gerard, 1931.

Oxygen consumption of fertilized eggs at different O_2 tensions. 228 to 80 mm. O_2, 100 per cent; 20 mm., 90 per cent; 13 mm., 80 per cent; 9 mm., 60 per cent; 4 mm., 40 per cent; 2 mm., 20 per cent. Below 11 mm. division rate slows; below 4 mm. division rate ceases[23] (Amberson, 1928). Temperature about 22°. 760 to 50 mm., 100 per cent; 28 mm., 80 per cent; 20 mm., 60 per cent; 15 mm., 40 per cent; 9 mm., 20 per cent (Tang and Gerard).[21] Temperature 25°.

For effect of methylene blue alone, with cyanide and with narcotics, see Barron, 1929.

For effect of CO_2 and HCl on oxygen consumption, see Root, 1930.

For oxygen consumption under various conditions see McClendon and Mitchell, 1912; Loeb and Wasteneys, 1911*b*, 1913, 1915; Wasteneys, 1916.

10. Oxidation-Reduction Potential

Not determined for *Arbacia* but from other sea urchin eggs. rH $= < 7.9$ in nitrogen and 12 ($E_0' = -0.06$ volts for pH $= 7$) (Chambers, Pollak and Cohen, 1929, 1931) or 21–22 (Needham, 1926) in air by indicator method. No change on fertilization and during segmentation.

Red pigment, echinochrome. $E_0 = +.1995$ volts at pH $= 0$, decreasing 0.06 volts for each pH unit except above pH $= 8.78$ where slope changes due to acid-base dissociation (Cannan, 1927).

11. Composition

Total nitrogen?

Total fat, carbohydrate and protein?

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[22] Winkler method.

[23] Haldane analysis of gas in equilibrium with sea water.

Lactic acid, 3.14 mg. per gram egg protein ($N_2 \times 6.25$). 81 per cent increase if treated with 0.003M KCN for 3 hours.[24] 19 per cent more lactic acid in 4 to 8-cell fertilized eggs (Perlzweig and Barron, 1928).

Free sugar absent (Perlzweig and Barron, 1928).

Reducing sugar by acid hydrolysis = 50 mg. glucose per gram egg protein (Perlzweig and Barron, 1928).

Total solid, 18.1 per cent (McClendon, 1909).

Ash, 8.5–10 per cent dry weight of eggs (Page, 1927).

Calcium,[25] 1.9 mg. per 10^6 eggs; 0.047 millimols; 10^6 eggs dried weigh 124 mg. (Page, 1927).

Magnesium,[26] 4.48 mg. per 10^6 eggs; 0.182 millimols (Page, 1927).

Sodium,[27] 1.301 mg. per 10^6 eggs; 0.056 millimols (Page, 1927).

Potassium,[28] 2.445 mg. per 10^6 eggs; 0.063 millimols (Page, 1927).

Iron,[29] 0.030 mg. per 10^6 eggs; 0.0005 millimols (Page, 1927).

Sulphate,[30] 0.00046 mg. per 10^6 eggs; 0.00004 millimols (Page, 1927).

Chloride,[31] 0.1864 mg. per 10^6 eggs; 0.0053 millimols (Page, 1927).

Total phosphate,[32] 0.9064 mg. per 10^6 eggs; 0.0291 millimols (Page, 1927).

Acid-soluble P, 0.40–0.50 mg. per 10^6 eggs; 0.0161 millimols (Page, unpublished).

Lipoid P, 0.498 mg. per 10^6 eggs; 0.160 millimols (Page, unpublished).

Nitrate, trace (Page, 1927).

Silica, moderate amounts (Page, 1927).

Copper, 17 micrograms per cc. eggs for unripe ovarian eggs; 175 micrograms per cc. for unfertilized and 21 micrograms per cc. for fertilized eggs (Glaser, 1923).

Cholesterol, present (Mathews, 1913).

Oil; iodine number 146–148; saponification value 606 (Page, 1927).

Echinochrome; see McClendon, 1912.

Catalase content of unfertilized and fertilized eggs same (Amberg and Winternitz, 1911); see Lyon, 1909.

Composition of layers of crushed and centrifuged unfertilized eggs (with jelly) in percentages of weight of whole egg mass (P = phosphorus, N = nitrogen) (McClendon, 1909).

[24] Nitrogen determined by micro-Kjeldahl and lactic acid by Clausen's method.

[25] Precipitated by NH_4 oxalate and ignited to CaO.

[26] Weighed as $Mg_2P_2O_7$.

[27] Kramer and Gittlemann pyroantimonate method.

[28] Kramer and Tisdall method.

[29] Allen-Scott colorimetric method.

[30] Weighed as $BaSO_4$.

[31] Mohr method.

[32] Benedict-Theis method.

Layer	Centripetal 32.5	Centrifugal 67.5 [33]	Whole Egg
Water	28.6	53.3	81.9
Solids	3.9	14.2	18.1
Ether ext.	0.308	1.946	2.254
P in ether ext.	0.00154	0.06760	0.06914
Alcohol ext.	1.60	3.48	5.08
P in alcohol ext.	0.0434	0.0814	0.1248
Water ext.	0.78	1.42	2.20
P in water ext.	0.130	0.182	0.312
Residue of water ext.	1.309	7.29	8.59
P in residue	0.0392	0.1167	0.1559
N in residue	0.1625	0.7750	0.9375
Ash in residue	0.0162	0.0264	0.0426
Total *P* in layers	0.21414	0.4478	0.66194

[33] Contains jelly.

Analysis of eggs washed with isotonic glucose (K. C. Blanchard, unpublished).

Ash	7–8 per cent dry weight of eggs
Calcium	6.45 ± 0.3 per cent of ash
Magnesium	1.77 ± 0.02 per cent of ash
Potassium	15.79 ± 0.25 per cent of ash
Lactic acid	0.4 mg. (when eggs first shed) to 6 mg. (on standing) per gram egg protein
Glycogen	50–80 mg. per gram egg protein

For analysis of autolysed eggs see Lyon and Shackell, 1910.

For egg secretions see Lillie, F. R., 1913; Glaser, 1914, 1921, 1922; Woodward, 1918; Clowes and Bachman, 1921.

12. Rate of Development

Time in minutes from fertilization to first cleavage (Loeb and Wasteneys, 1911*a*).

° C.	Time	° C.	Time	° C.	Time
7	498	16	85	26	33
8	410	17.5	70	27.5	34
9	308	18	68	30	33
10	217	20	56	31	37
12	147	22	47	32	No cleavage
15	100	25	40	35	No cleavage

° C.	Q_{10}	° C.	Q_{10}	° C.	Q_{10}
7–17	7.3	10–20	3.9	15–25	2.6
8–18	6	12–22	3.3	16.26	2.6
9–19	> 4	13–23	3.3	17.5–27.5	2.2
				20–30	1.7

Time from fertilization to 50 per cent first cleavage, 62.5 minutes at 20.6–20.8° C. (Haywood and Root, 1930); 55 minutes to 50 per cent first cleavage and 83 minutes to second cleavage at 22° C. (Lillie and Cattell, 1923); 50 minutes to 50 per cent first cleavage at 22° C. (Whitaker, 1929).

Time from fertilization at 23° C. to various stages of development (E. B. Harvey).

Completion of fertilization membrane, 1 to 2 minutes.
Hyaline plasma layer begins 2 minutes.
Union of pronuclei (monaster), 10 minutes.
Nuclear streak begins 20, ends 40, marked 25–30 minutes.
Nuclear membrane disappears, 40 minutes.
Amphiaster, 45 minutes.
First cleavage (50 per cent), 50 minutes.
Second cleavage (50 per cent), 78 minutes.
Third cleavage (50 per cent), 103 minutes.

For effect of sea water diluted with .73M cane sugar see Lillie and Cattell, 1923.

For effect of CO_2 and acids on development see Clowes and Smith, 1923, Smith and Clowes, 1924, *a* and *b*, and Smith, 1925.

For effect of CO_2 on cleavage rate see Haywood, 1927, and Haywood and Root, 1930.

For effect of lack of oxygen see Harvey, E. B., 1930, and Lyon, 1902.

For effect of $HgCl_2$ see Hoadley, 1930.

For effect of mechanical shocks see Whitney, 1906.

For cleavage rates of centrifuged egg fragments see Harvey, E. B., 1932*a*, and Whitaker, 1929.

13. NARCOSIS

Anaesthetic and Lethal Concentrations (Heilbrunn, 1920) of

	Anaesthetic *per cent*	Lethal *per cent*
Ether	2	4
Chloroform	0.13	1
Chloral hydrate	0.25	1
Nitromethane	2	3
Paraldehyde	4	8
Acetone	5	10
Ethyl nitrate	0.4	?
Ethyl acetate	2	5
Ethyl butyrate	0.25	0.5
Acetonitrile	4	5
N Propyl alcohol	1	?
Amyl alcohol	0.6	1
Phenyl urethane	4/5 sat.	sat.
Ethyl urethane	1.5	3

Critical narcotic concentrations for reversibly suppressing cleavage (Harvey, E. B., 1932*b*).

Ethyl urethane	0.15–0.2 M
N-propyl urethane	0.07 M
Isopropyl urethane	0.1 M
N-butyl carbamate	0.025 M
Isoamyl carbamate	0.01 M
Phenyl urethane	0.00125–0.0025 M

Critical narcotic concentrations for reversibly suppressing cleavage (Lillie, 1914).

Methyl urethane	2–2.5 per cent; .29–.33 M
Ethyl urethane	1.5–1.75 per cent; .15–.19 M
Phenyl urethane	.08–.06 per cent; .005–.006 M
Ethyl alcohol	5 vol. per cent; .87 M
Propyl alcohol	2 vol. per cent; .27 M
Isopropyl alcohol	3 vol. per cent; .4 M
H-Butyl alcohol	.8 vol. per cent; .086 M
Isoamyl alcohol	.4 vol. per cent; .037 M
Capryl alcohol	.015 vol. per cent; .001 M
Ethyl ether	.5–.6 vol. per cent; .05–.06 M
Chloroform	.06 per cent; 1/12 saturated; .005 M
Chloral hydrate	.1–.12 per cent; .006–.007 M
Chloretone	.2–.25 per cent; .008–.01 M

See also Baldwin, 1920, for effect of alcohols on dividing eggs.

14. Miscellaneous

For **effects of light** see Lillie and Baskervill, 1921, 1922, and Hinrichs, 1926, 1927.

For **effect of salts** see Lillie, R. S., 1910, 1911; Lillie and Baskervill, 1921.

For **effect of supersound waves** see Harvey, Harvey and Loomis, 1928, and Harvey and Loomis, 1931.

For **rhythms of susceptibility during development** see Lyon, 1904, Baldwin, 1920, and Page, 1929; Lillie, R. S., 1916 and Just, 1928, for hypotonic sea water; Moore, A. R., 1915, for hypertonic sea water.

For **initiation of development** and related phenomena see Greeley, 1902; Hunter, 1903; Harvey, E. N., 1910; Loeb, 1913, 1915; Heilbrunn, 1913, 1915, 1920, *a* and *b*, 1925; Lillie, F. R., 1914, 1921; Glaser, 1914; Moore, C. R., 1916, 1917; Just, 1922, 1928, *a* and *b*, 1929.

For **surface precipitation reaction** see Heilbrunn, 1930.

For **agglutination** see Goldforb, 1929.

For **nature of fertilization membrane** see Kite, 1912; Heilbrunn, 1913, 1915, 1924*a*; Harvey, E. N., 1914; McClendon, 1914; Garrey, 1919; Chambers, 1921.

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THE DEVELOPMENT OF HALF AND QUARTER EGGS OF ARBACIA PUNCTULATA AND OF STRONGLY CENTRIFUGED WHOLE EGGS

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When the unfertilized eggs of *Arbacia punctulata* are strongly centrifuged in a sugar solution of the same (or graded) density, they become dumb-bell shaped and then separate into two nearly equal spheres or half-eggs, one colorless and the other pigmented (E. N. Harvey, 1931). Further, when the colorless spheres are again centrifuged, more strongly, in a sugar solution of the same (or graded) density, they separate into two spheres or quarter-eggs, one without visible granules and the other with granules. All of these half- and quarter-eggs, as well as the deformed total eggs just before pulling apart, can be fertilized and develop. We can in this way obtain "egg fragments" of very definite size and content in great numbers. This obviates the former tedious method of cutting individual eggs with a glass needle (Harnley, 1926; Hörstadius, 1928; Plough, 1929; Tennent, Taylor and Whitaker, 1929; Whitaker, 1929) and insures a much more accurate division of the egg into parts of known structure than the old haphazard and harmful method of shaking the eggs into pieces (Hertwigs, Boveri, Driesch, Morgan, etc.).[1] Sea urchin eggs centrifuged in sea water alone do not separate into spheres owing to the fact that they sink to the bottom and are crushed by the force exerted on them. The method employed was to put two parts 0.95 molal sugar (95 per cent of 342 grams cane sugar added to 1 liter tap water, an isotonic solution) in the centrifuge tubes and above this one part of sea water containing eggs; the tubes were rolled gently to obtain partial mixing and then centrifuged at about 7000 r.p.m. (11 cm. radius) for four minutes. On removal of the tubes, the colorless spheres formed a whitish layer just beneath the surface, the whole eggs, which had not broken apart, formed a reddish layer about halfway down the tube and the pigmented spheres rested at the bottom of the tubes. One could then pipette off any one of the three kinds of eggs with practically no admixture of the other varieties. The sugar solution was found to have no ill effect on the eggs, since eggs could be kept in this solution for at least five hours and when returned to sea water could be fertilized, and developed normally. Eggs cannot be fertilized, however, while in the sugar solution although the sperms are active and surround the egg. The rapid centrifuging also has no ill effect upon the eggs, as eggs subjected to this treatment develop into normal plutei when fertilized in sea water. To separate the colorless half-eggs into two parts, they were taken from the centrifuge tubes and put into other centrifuge tubes above a small amount of cane sugar solution (3 parts of 0.95 molal sugar to 1 part sea water) and a little sea water placed above. The tubes were then centrifuged for 45 minutes at about 10,000 r.p.m. (radius 11 cm.)

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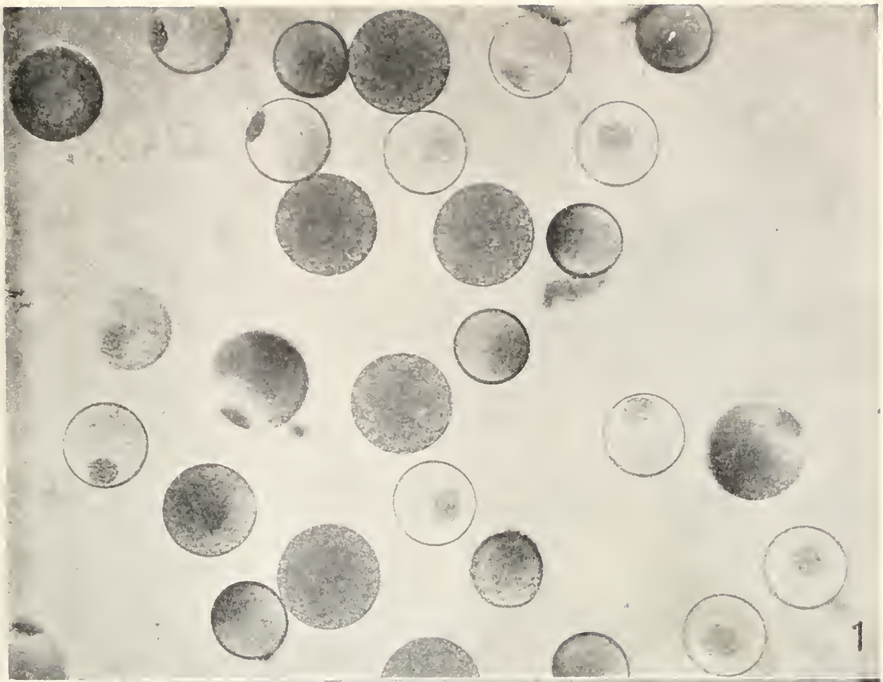
[1] The centrifuge method of separating eggs supplements rather than supplants the cutting method, since the granular constitution of fragments is different in the two cases.

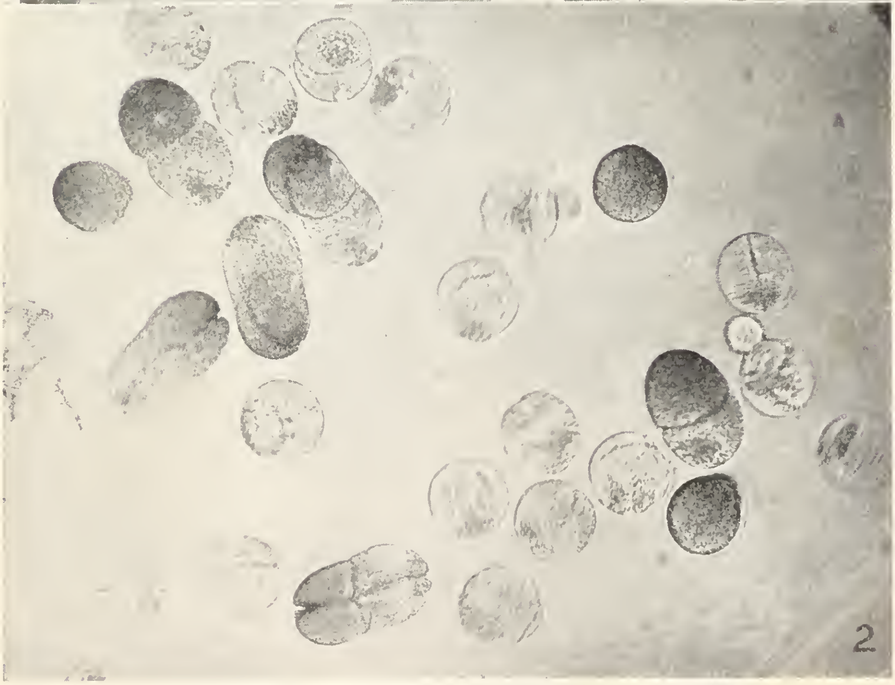
Unfertilized Whole, Half- and Quarter-eggs

When normal *Arbacia* eggs are centrifuged as described above, they become stratified while still spherical into five layers, (1) oil on top, (2) a clear layer without visible (*i.e.*, under ordinary illumination) granules, in which lies the nucleus, (3) a thin granular layer separated out only with strong centrifugal force, and not heretofore described and which we have, therefore, termed "the fifth layer," (4) a large yellowish yolk layer, and (5) a layer of red pigment concentrated at the heavier pole (Fig. 25). The fifth layer can be very beautifully demonstrated by staining with methyl violet or methyl green when it becomes purple, or with Janus green when it becomes green.

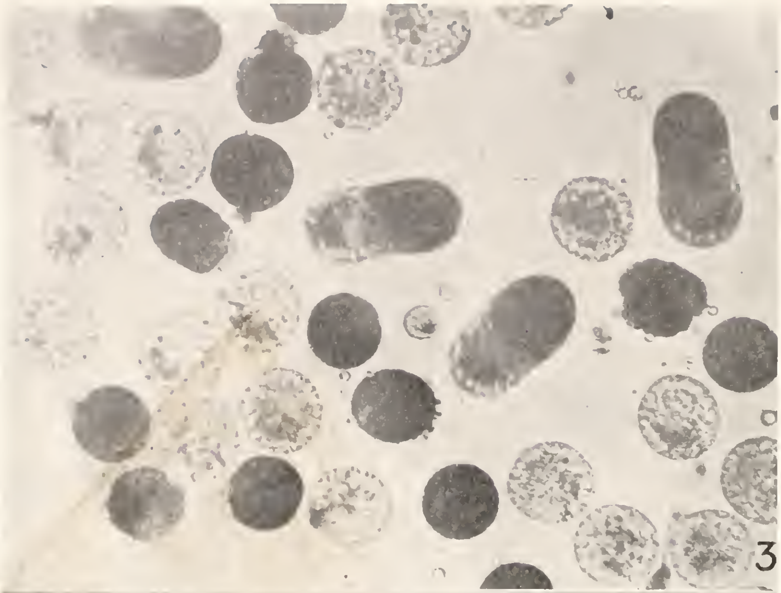
The eggs after stratification become elongate, then dumb-bell shaped (Fig. 25) and then break into two slightly unequal spheres, usually through the upper part of the yolk. The colorless sphere (cf. Fig. 1), slightly larger, contains therefore the oil, below which always lies the nucleus; the clear layer, without visible granules; the granular or fifth layer; and a little yolk at the heavier pole. The pigmented sphere, (cf. Fig. 13) slightly smaller, contains usually only yolk and pigment, the pigment being massed at the heavier pole; there is, of course, no nucleus in this sphere. In a few batches of eggs, the two spheres were almost equal in size, the separation having taken place in the fifth layer, a little of which appeared as a light cap on the pigmented sphere. Usually, however, there is quite an appreciable difference in size of the two half-eggs, and their relative size is fairly constant in any one centrifuged lot. When the colorless spheres are centrifuged again, as described above, they separate into (1) a larger sphere with oil, nucleus and clear layer—this quarter-egg now having practically no visible granules (Fig. 38); and (2) a quite small sphere composed entirely of the granules of the fifth layer and yolk, about half of each (cf. Fig. 46).

The average measurements obtained in typical lots of eggs are given in Table I. A photograph of unfertilized whole eggs and the two





Photograph 1. Unfertilized whole and half-eggs.
Photograph 2. 2 and 4-cell whole and colorless half-eggs.



Photograph 3. Blastulae of whole and colorless half-eggs.
Photograph 4. Five types of eggs, whole, half- and quarter-eggs.

kinds of half-eggs is given in Photograph 1. Photograph 4 is a picture of the five types of eggs, whole, half- and quarter-eggs.

Development of Half-eggs

Both the colorless and pigmented half-eggs can be fertilized in sea water immediately after centrifuging or at any later time. They both form fertilization membranes at the same time as the whole egg. These are often well separated from the surface of the egg but sometimes, especially in the pigmented spheres, rather closely investing. There is in both half-eggs a well marked ectoplasmic or hyaloplasmic layer formed on fertilization. The development of the two half-eggs must now be followed separately. All observations were made on living material, and the times given are for 23° C., when 50 per cent normal eggs cleave in 50 minutes.

The development of the colorless half-egg is quite normal. Many of the nuclear phenomena accompanying fertilization and cleavage can be seen with great clearness, and they parallel those described by Wilson (1895) in the living *Toxopneustes* egg, which is devoid of pigment and quite transparent; these phenomena cannot be observed in the normal living *Arbacia* egg on account of the pigment. The sperm aster can be seen in the granular area a few minutes after fertilization, and its approach toward the female pronucleus can be followed (Fig. 2). The female pronucleus travels down from its position under the oil cap toward the center of the egg about eight minutes after fertilization (Fig. 2), and as it moves down some of the oil spherules also move down from the oil cap. After the union of the pronuclei (Fig. 3) astral radiations extend through the granular area; in fact all the granules are often arranged in rays extending in a half circle from the nucleus (Fig. 4); there is no indication of rays (in the living egg) in the clear area free of granules. In the normal uncentrifuged egg at this period, the astral radiations extend throughout the cell, this being the "monaster stage" (Fig. 36). These radiations

TABLE I

	Diameter	Volume	Sums of Volumes
	μ	μ³	μ³
Whole Egg	73	203700	
Colorless Half Egg	60	113100	205050
Pigmented Half Egg	56	91950	
Clear Quarter Egg	53	77950	111450
Granular Quarter Egg	40	33500	

gradually fade out and the nucleus enlarges from a diameter of 12 μ to 16 μ (Fig. 5). This is the "streak stage" of the normal *Arbacia* egg, lasting from 20 to 40 minutes after fertilization, characterized by a curved band extending on either side of the nucleus (Fig. 37); this stage is referred to by Wilson in the *Toxopneustes* egg as the "pause." The nuclear wall now breaks down (Fig. 6) and soon afterwards there are again radiations, now from the two poles of the amphiaster, but present only in the granular zone (Figs. 7, 8). The half-eggs divide about the same time as normal control eggs, or sometimes a little earlier as Whitaker (1929) found for his diploid fragments; the plane of cleavage usually comes in perpendicular to the stratification (Fig. 9), but sometimes parallel with it (Fig. 10) or in any intermediate position, but it divides the egg into two equal parts (photograph 2) as in the normal egg. The following cleavages come in at right angles to the preceding and divide the blastomeres equally (Figs. 11, 12). Blastulae (photograph 3) are formed from the half-eggs, quite normal except for size and coloring. In many of the cultures the larvae remained for several days as very actively swimming blastulae; in some they developed into gastrulae and in some into plutei with well developed skeleton and arms exactly like the normal ones except that they were colorless and only half the size.

The pigmented spheres have no nucleus at the time of fertilization. The aster accompanying the sperm nucleus may very often be seen in the yolk 15–20 minutes after the fertilization membrane has been given off (Fig. 13). The nucleus, at first very small, enlarges and is quite noticeable about 30 minutes after fertilization (Fig. 14), and about the time of first cleavage of the whole and colorless half-eggs. This single nucleus now usually without radiations, after enlarging considerably (Fig. 15), breaks down about one hour after fertilization (Fig. 16). At about 80 minutes after fertilization (Fig. 19), when the total and colorless spheres are in the 4-cell stage, many of the pigmented spheres have two nuclei; either a dumb-bell shaped nucleus or a very small amphiaster can sometimes be seen preceding the binucleate stage (Figs. 17, 18). Usually no cleavage plane comes in, probably owing to the mechanical difficulty in cutting through the dense material. After several successive divisions of the nuclei, the pigmented half-eggs appear as reddish spheres containing many white circles, some with radiations, giving somewhat the appearance of pictures of the moon with craters on the surface (Fig. 20). There are often slight indications of cleavage planes running in from the periphery as indentations or notches (Figs. 21, 22). Rather rarely, the division planes come in quite normally and the egg divides into 2, 4 and 8 equal blastomeres

(Figs. 23, 24). The first cleavage plane in these cases may come in in any relation to the stratification, and the following planes at right angles to the preceding. Although it is difficult to determine in living material, it would seem that when cleavage planes do not come in at first, they come in later on after some 20 or 30 nuclei are formed, for the blastulae seem quite normal and appear, as far as I could tell, multicellular. The blastulae are quite active, but are not very viable, and relatively few in any batch develop much further. I have, however, had a number of quite normal gastrulae and some plutei with skeletons, but only two with well developed arms. None have survived more than nine days. There seems no doubt that these merogonic eggs *can* give rise to dwarf embryos similar to normal ones except for containing more pigment. Whether the failure of the majority of these eggs to develop far is due to lack of certain formative stuffs or to the lack of the female pronucleus, or to the over-crowding with dense material is not certain, but I should judge from the appearance and behavior of the developing eggs that the last explanation is the correct one. It may be, however, that only those half-eggs containing some of the fifth layer develop, but this awaits further investigation.

It would seem then that both half-eggs can develop into plutei, and that neither food material (or very little) nor a female pronucleus is necessary for development.

Development of Strongly Centrifuged Whole Eggs

When the unfertilized *Arbacia* eggs are strongly centrifuged, as mentioned before, they elongate, then become dumb-bell shaped before breaking apart (Fig. 25). When these are left either in the sugar solution or in sea water, they soon become oval and then spherical, often within an hour. But if they are removed to sea water and fertilized immediately, they retain their dumb-bell shape and the fertilization membrane follows the contour of the surface with usually a bulge at one or both poles. This must be due to a "setting" or gelation of the protoplasm following fertilization, for if the fertilization membranes are removed by drawing the eggs into a capillary pipette, the eggs retain their aspherical shape (and develop). This is another indication of increased viscosity following fertilization. One can often observe the male aster in the yolk or granular zone, and this apparently pulls some pigment granules along as it travels toward the center (Figs. 26, 27). The descent of the female pronucleus can be observed and the pull of the oil spheres toward the center, the union of the two pronuclei and the gradual fading out of the sperm aster whose radiations have been visible only in the granular zone (Fig. 27), the enlarge-

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ment of the nucleus and its rupture (Figs. 29, 30). A very striking stage is shown in Fig. 28, a sharp line of demarcation running between the clear zone and the granular zone; this corresponds to the "streak" stage in the normal egg (Fig. 37). A streak is only rarely seen running perpendicular to the stratification in well centrifuged eggs, probably owing to the denseness of the material. Later, rays from the amphiaster are seen in the yolk and granules of the fifth layer, which by this time are thoroughly mixed (Fig. 31); either a whole aster whose mate is sometimes faintly distinguishable in the clear zone into which some granules have spread, or less commonly two half asters in the granular and yolk zone. It would seem that there must be a change of axis in the mitotic figure, for in normal eggs the long axis of the spindle is the same as the long axis of the "streak," whereas here it is usually perpendicular to it. The first cleavage plane comes in at almost the same time as in normal eggs, usually through or near the constriction and parallel (or at a slight angle) with the stratification, separating one colorless blastomere from the one containing yolk and pigment (Fig. 32). These two cells are usually unequal in size, more frequently the colorless cell is the smaller, though the two blastomeres are sometimes of size corresponding with the two half-eggs, the cleavage plane following the future separation plane (Photograph 2). The first cleavage plane comes in rarely in these elongated eggs perpendicular to the stratification, and this is sometimes followed by a second cleavage plane also along the long axis resulting in four sausage shaped cells. The second cleavage plane always comes in perpendicular to the first (Fig. 33, Photograph 2). Many years ago, Lyon (1907) and Morgan and Lyon (1907) found that the first cleavage plane in centrifuged *Arbacia* eggs was usually perpendicular to the stratification. The apparent contradiction is explained by the difference in shape of the eggs. In *elongated* eggs, the first cleavage plane comes in usually parallel with the stratification, in the short axis. In *spherical* eggs it comes in perpendicular with the stratification; this is true both for eggs which are spherical because not centrifuged sufficiently to become elongate, and for eggs which have been elongate but have resumed a spherical shape on standing in sea water before fertilization. In slightly elongate or oval shaped eggs, the first cleavage plane comes in one way or the other in about equal numbers.

The later cleavage planes often come in fairly regularly except that the clear cells often divide in advance of the pigmented cells, and are smaller (Fig. 34). Very frequently, the original first cleavage plane remains quite prominent, the first two blastomeres developing almost independently (Fig. 35). So much so, in fact, that double embryos are often produced, one colored and the other colorless, at first within the same membrane, and later swimming attached together. Slipper shaped blastulae (Photograph 3), normal gastrulae and plutei arise from the elongate and dumb-bell shaped eggs, but the stratification of pigment and yolk remains, and may be in any relation, apparently, to the axis of the embryo. Individual eggs have not been studied with reference to polarity.

Development of Quarter Eggs

The colorless half-eggs, when centrifuged again, are drawn out into dumb-bells (Fig. 51) and are then separated into spheres. The stratification is the same as before, the nucleus lying just below the oil cap in a large clear layer without visible granules; at the heavier pole is the granular or fifth layer and a layer of yolk. The half-egg breaks usually at the line between the clear layer and the granules, so that we obtain one perfectly clear sphere, with oil cap and nucleus (Fig. 38) and one granular sphere containing about an equal amount of granules (fifth layer) and yolk (cf. Fig. 46). This granular quarter-egg is much smaller than the clear quarter, about one sixth the volume of the whole egg.

Both of these quarter-eggs, as well as the dumb-bell shaped half-eggs can be fertilized in sea water, and throw off fertilization membranes. The ectoplasmic layer of the granular quarter is much thicker than that of the clear quarter, where it is thinner than in normal eggs.

Owing to the absence of granules in the clear quarter, nothing can usually be seen of the sperm aster; the female pronucleus can be observed migrating from the oil cap to the center of the egg about 25 minutes after fertilization, pulling some of the oil spheres along. This gradually enlarges from 12 μ to 16 μ, but no other change occurs for six hours or more (Fig. 39). In some eggs the nucleus becomes enormous, as large as 22 μ (nearly half the diameter of the egg, and an increase of six times in volume), but this is probably abnormal. The nucleus later breaks down and disappears (Fig. 40) and cleavage takes place some seven hours (or more) after fertilization (Figs. 41, 42) and in any plane with regard to the oil cap. The very slow cleavage of these diploid quarters is not in accord with Whitaker's (1929) explanation of cleavage rates. Other cleavages follow slowly, but usually the membrane breaks and there is a loose mass of cells, some perfectly clear and some with oil drops (Figs. 43, 45). I have obtained a few intact later cleavages, particularly in eggs left for several hours after recentrifuging before fertilizing them (Fig. 44).

The granular quarter-eggs (Fig. 46), on the other hand, although having no female nucleus, develop quite normally. The male aster can be seen in the granules (Fig. 47), the nucleus enlarges, disappears, and the egg cleaves a little later than the control eggs (Fig. 48). This then divides into 4, 8, 16 approximately equal cells usually retaining the fertilization membrane (Figs. 49, 50).

Whether the quarter-eggs could give rise to swimming plutei will be investigated further. This part of the work was done for a short period late in September when the eggs are not in the best condition. The granular quarters went as far as the stage just before they become free-swimming and looked quite normal at that time.

The recentrifuged half-eggs which have become dumb-bell shaped (Fig. 51) retain their shape if fertilized immediately, and round up if left unfertilized just as the whole eggs do. The rounded eggs develop in the same way as before recentrifuging. The dumb-bell shaped half-eggs form a fertilization membrane following their contour and the nuclear phenomena accompanying fertilization can be clearly seen (Figs. 52–54). The first cleavage usually comes in near the junction of the clear with the granular area, giving one clear cell with oil cap and one granular cell (Fig. 55). The granular cell often precedes the clear cell in division just as it does when completely separate. The fertilization membrane usually breaks after several divisions, giving a loose mass of cells, some clear and some granular (Figs. 56–59).

Micro-dissection

The difference in the material of the white and red half-eggs can be well demonstrated by micro-dissection. When the colorless half-eggs are punctured by a needle they immediately explode, the granules and nucleus flowing out and leaving the membrane empty. When the pigmented half-eggs are punctured, there is no flow of granules, the material is quite pliable and elastic; it can be pulled out in strands which will go back again and resume a spherical form, or it can be cut in parts, each of which may round up. The stratified whole egg responds in a similar way. When the clear zone is punctured, the granules flow out. When the yolk or red layers are punctured, the material can be pulled out and released without any explosion or loss of material, and it behaves like an elastic and pliable substance. When the clear quarter-egg is punctured, it explodes immediately. When the granular quarter is punctured, the granules flow out but quite slowly.

Parthenogenesis

The question of parthenogenesis in the half- and quarter-eggs has been studied only slightly. Just (1928), and others previously, found that by treating unfertilized *Arbacia* eggs for a few seconds with distilled water and then returning them to sea water, the eggs formed fertilization membranes and developed to the stage just before cleavage. When the half- and quarter-eggs are thus treated, they all form beautiful fertilization membranes and good ectoplasmic layers. In the centrifuged whole eggs and in colorless half-eggs, the nucleus descends toward the center of the egg just as in fertilized eggs; this then is *not* an attraction by the male pronucleus. The nucleus enlarges and breaks just as in fertilized eggs. Astral radiations characteristic of fertilized eggs at the time of union of the pronuclei are, of course, absent, but the astral radiations from the amphiaster are later seen in the granular zone. In the pigmented half-eggs, no development further than the formation of the fertilization membrane and ectoplasmic layer has been observed, nor would it be expected since there is no nucleus of any sort. The clear quarters start to develop just as the colorless half-eggs, as indicated by the descent of the nucleus to the center. The granular quarters, like the pigmented half-eggs, show no further development after the formation of the fertilization membrane and ectoplasmic layer.

SUMMARY

1. With strong centrifugal force and the proper medium, *Arbacia* eggs can be separated into two half-eggs, one colorless containing oil, nucleus, clear layer, fifth (granular) layer and a little yolk; the other slightly smaller containing yolk and pigment. With greater centrifugal force, the colorless half-eggs can be separated into quarter-eggs, one perfectly clear with oil and nucleus; the other, smaller, with fifth layer (granules) and yolk. All of these half- and quarter-eggs can be fertilized, form fertilization membranes and cleave.

2. Nuclear phenomena accompanying fertilization and cleavage, quite normal, can be observed with great clearness in the colorless half-eggs. Astral rays occur only where granules are present. These half-eggs cleave regularly and form swimming blastulae and plutei, normal except for color and size.

3. The pigmented half-eggs develop with only the male nucleus which divides repeatedly, usually without cell division. Some blastulae and a few plutei developed but these eggs and larvae are not very viable.

4. Whole eggs, centrifuged till dumb-bell shaped, retain their shape if fertilized immediately, even if the fertilization membrane is removed. The first cleavage in elongate eggs is usually parallel with the stratification, in spherical eggs it is usually perpendicular to it. Slipper shaped blastulae develop from the elongate eggs, and normal plutei.

5. Clear quarter-eggs begin to cleave very slowly (after 7 hours), and usually form loose clusters of cells owing to the breaking of the fertilization membrane.

6. Granular quarter-eggs develop with only the male nucleus and a little more slowly than the normal whole eggs; cleavage is quite regular but no swimming blastulae were obtained.

7. The pigmented half-eggs can be drawn out with a microdissection needle, and the material is pliable and elastic; the colorless half-eggs explode when punctured, pouring out granules. The clear quarter-eggs collapse immediately when punctured, and the granular quarters pour out their granules slowly.

8. All of the half- and quarter-eggs will start to develop parthenogenetically, *i.e.*, throw off a fertilization membrane, if treated with distilled water. Only those with a nucleus develop further, till just before cleavage.

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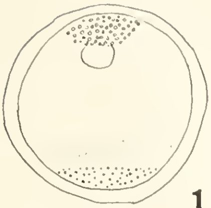
DESCRIPTION OF PLATES

The drawings have been made from living eggs entirely, and are magnified 266 X. The large solid dots represent pigment, the coarse stippling yolk granules, fine stippling the granules of the fifth layer, small circles oil drops. The times given are times after fertilization, approximate for 23° C. (controls cleave in 50 minutes).

FIGS. 1–12. Colorless half-eggs; Figs. 13–24 pigmented half-eggs; Figs. 25–35 centrifuged whole eggs; Figs. 36, 37 normal whole eggs; Figs. 38–45 clear quarter-eggs; Figs. 46–50 granular quarters; Figs. 51–59 recentrifuged colorless half-eggs.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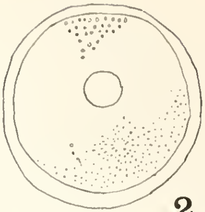
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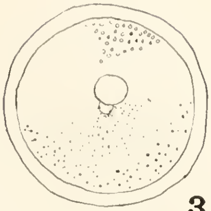
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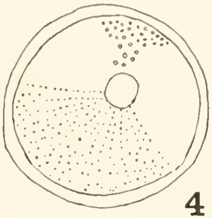
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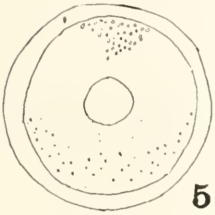
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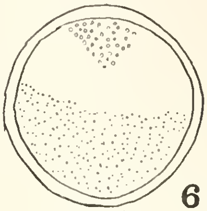
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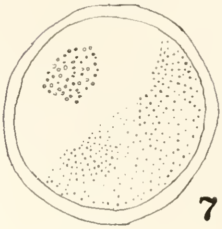
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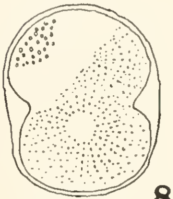
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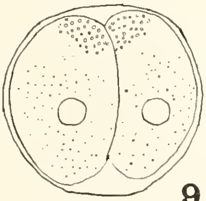
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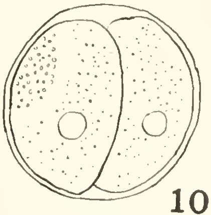
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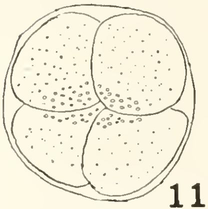
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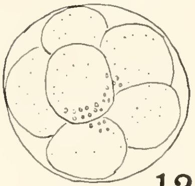
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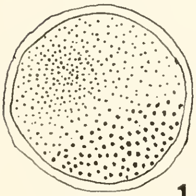
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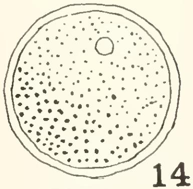
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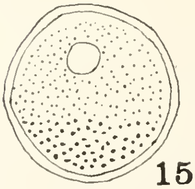
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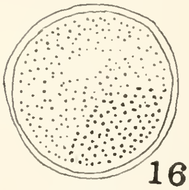
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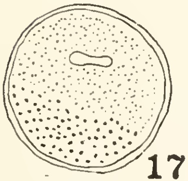
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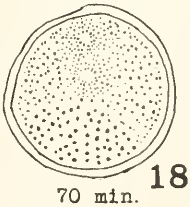
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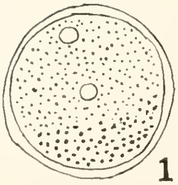
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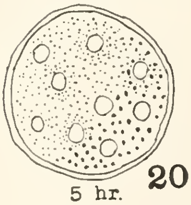
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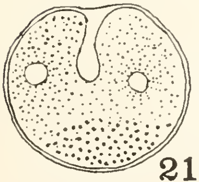
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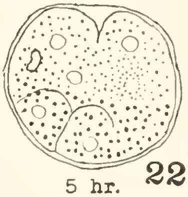
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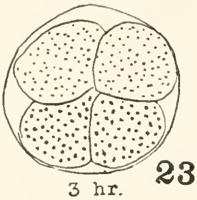
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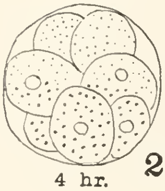
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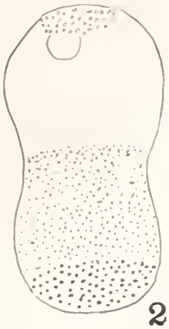

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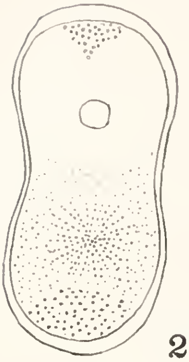

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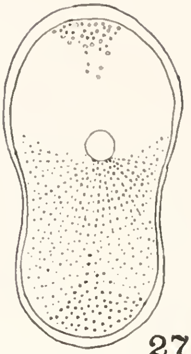

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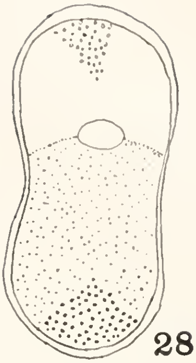

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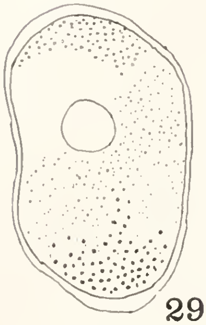

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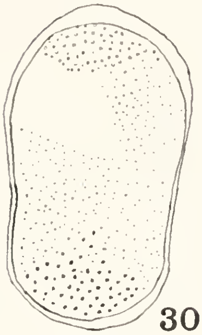

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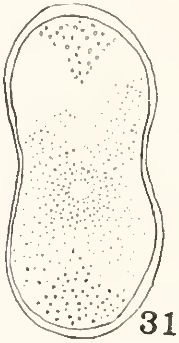

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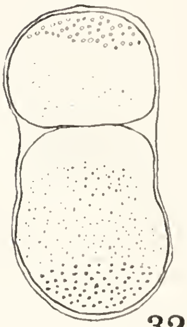

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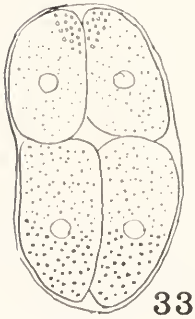

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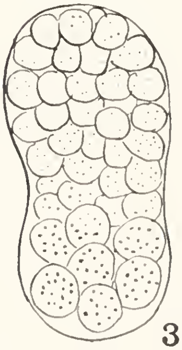

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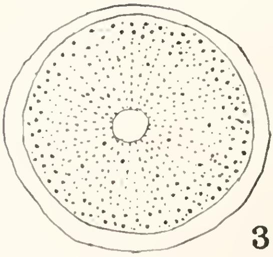

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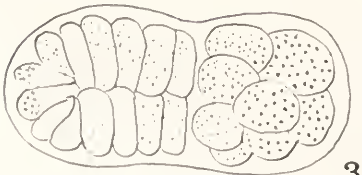

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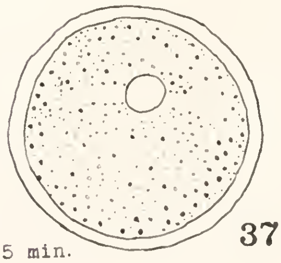

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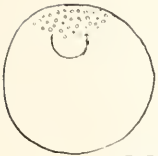
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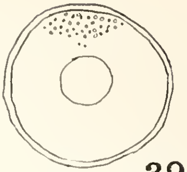
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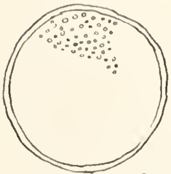
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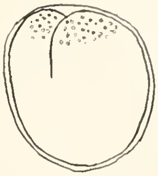
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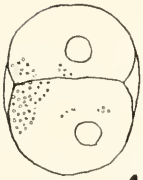
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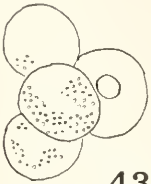
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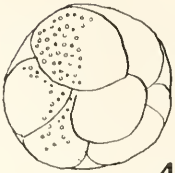
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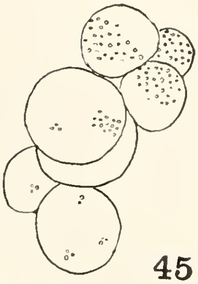
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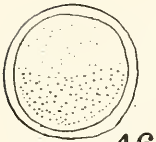
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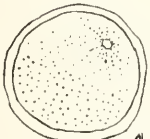
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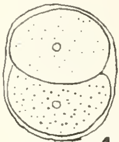
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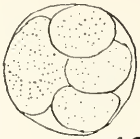
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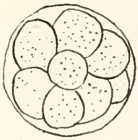
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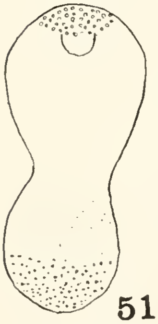
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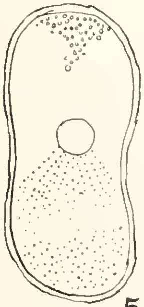
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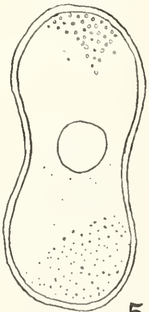
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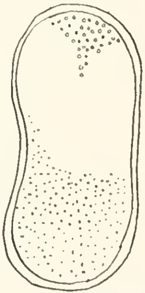
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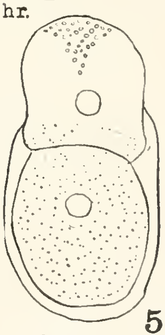
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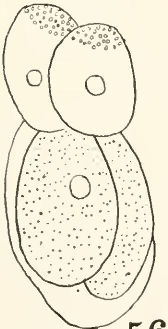
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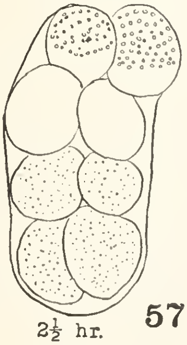
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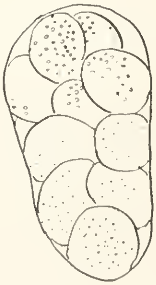
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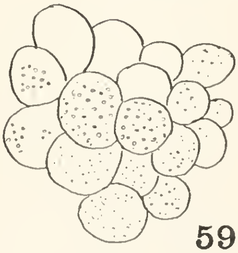

4 hr. **59**

MIGRATION OF THE PROXIMAL RETINAL PIGMENT IN THE CRAYFISH IN RELATION TO OXYGEN DEFICIENCY

RUDOLF BENNITT AND AMANDA DICKSON MERRICK
Zoölogical Laboratory, University of Missouri

The primary factors determining the position of the pigment in the proximal and distal sets of pigmented cells of the crustacean retina (Fig. 1) are light and darkness. Whether they act directly on the cells or indirectly via the circulation or the nervous system has not been definitely determined. But ever since Exner (1891) called attention to the optical importance of ensheathing the visual axis (Fig. 1, rhabdome and cone) of the ommatidium with opaque pigment in the light and of withdrawing this sheath in darkness, thereby allowing the photoreceptor (the rhabdome) to make use of light other than that which enters along its axis, most investigators have regarded this as a more or less adaptive mechanism, though of somewhat doubtful effectiveness.

It became apparent, however, that under some circumstances factors other than light and darkness might induce changes in the proximal and distal sets of pigmented cells of the crustacean retina. For example, Congdon (1907) found that temperature was such a factor in certain Crustacea. Demoll (1911) and Bennitt (1924) found that anaesthesia and death, in noctuid moths and crustaceans respectively, were always accompanied by the extreme "light-adapted" position of the retinal pigment, regardless of the surroundings. Welsh (1930*a*) recently noticed that anaesthesia had this effect on the distal retinal pigment of the shrimp *Macrobrachium*. He further observed a "diurnal rhythm" in the migrations of the distal pigment cells in the retina of this animal, even under constant illumination, and this he believed to be associated with the same sort of "metabolic periodicity" to which had been ascribed numerous other cases of periodic change—in color, luminescence, etc. Bennitt (1932*b*) observed a similar, though less extensive, diurnal rhythm in the proximal pigment cells of the retina in crayfishes which were kept for several days in total darkness. Finally, Bennitt (1924, 1932*a*) obtained evidence that under certain conditions stimulation of one eye might result in bodily changes affecting the position of the proximal pigment in the other (unstimulated) eye; whether these changes are nervous or vascular or both is still undecided.

It would seem, therefore, that in addition to the usual photic changes associated with the action of the proximal pigmented cells of the retina there is an internal mechanism of some sort whose operation may, under certain conditions, bring about changes which were at first thought to be solely photomechanical. The experience of Demoll, Bennitt, and Welsh with anaesthetics, referred to above; Arey's (1916) discovery that oxygen deficiency was associated with retraction of the

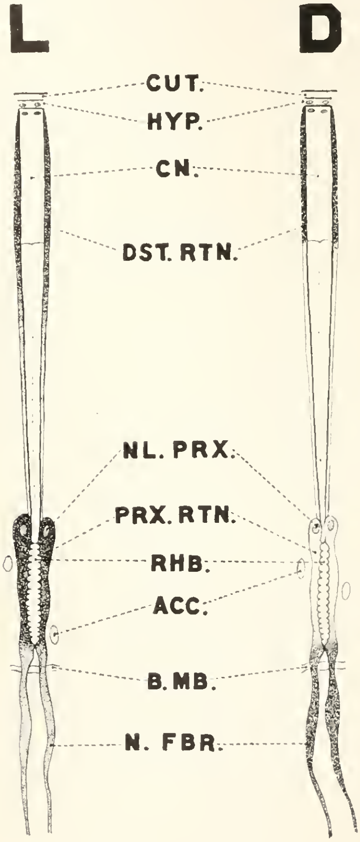


FIG. 1. Longitudinal sections of two ommatidia of *Cambarus virilis* Hagen, showing the arrangement of retinal pigment in the light-adapted (*L*) and dark-adapted (*D*) conditions. Only two of the seven proximal retinal pigment cells are shown in each ommatidium.

ACC., nuclei of accessory pigment cells; *B.MB.*, basement membrane; *CN.*, cone; *CUT.*, cuticle; *DST.RTN.*, distal retinal pigment cells; *HYP.*, cells of the hypodermis; *N.FBR.*, prolongations of the proximal retinal cells which form nerve-fibers into the optic ganglia; *NL.PRX.*, nuclei of proximal retinal pigment cells; *PRX.RTN.*, proximal retinal pigment cells; *RHB.*, rhabdome.

× 125.

retinal pigment in fishes; the work of Spaeth (1913) on the melanophores of fishes and of Uyeno (1922) on those of the frog, both of which showed distinct pigmentary changes accompanying oxygen want; and some early observations of the writers and others on the effect of overcrowding crayfish in an aquarium—all these led us to investigate the specific effect of oxygen deficiency on the action of the retinal pigment cells of the crayfish. We limited this investigation to observations on the *proximal* pigment cells, in which pigment streams distally in the light and proximally in darkness through the cytoplasm of cells of virtually fixed length (cf. Fig. 1 and Plate 1). Photomechanical movements of the distal cells have been described in the shrimp *Palaemonetes* by Welsh (1930*b*).

When a crayfish is "light-adapted" (Fig. 1, *L*; Plate 1, *L*), anaesthesia or death evoke no change in the position of the proximal pigment. When, however, the crayfish is "dark-adapted" (Fig. 1, *D*; Plate 1, *D*), death, anaesthesia, or simply overcrowding in the aquarium induce distal migration of the proximal retinal pigment even though the animal is in total darkness throughout the experiment.

GENERAL METHODS

The crayfishes, *Cambarus virilis* Hagen and *C. clarkii* Girard, were killed in hot water to fix the position of the pigment, and were examined: (1) after sectioning in paraffin, or (2) after macerating for 18 hours or more in Bela Haller's fluid. The latter method was simpler, and was equally reliable for the study of proximal cells, especially since the rhabdomes and accessory pigment cells usually adhered to the dissociated proximal pigment cells.

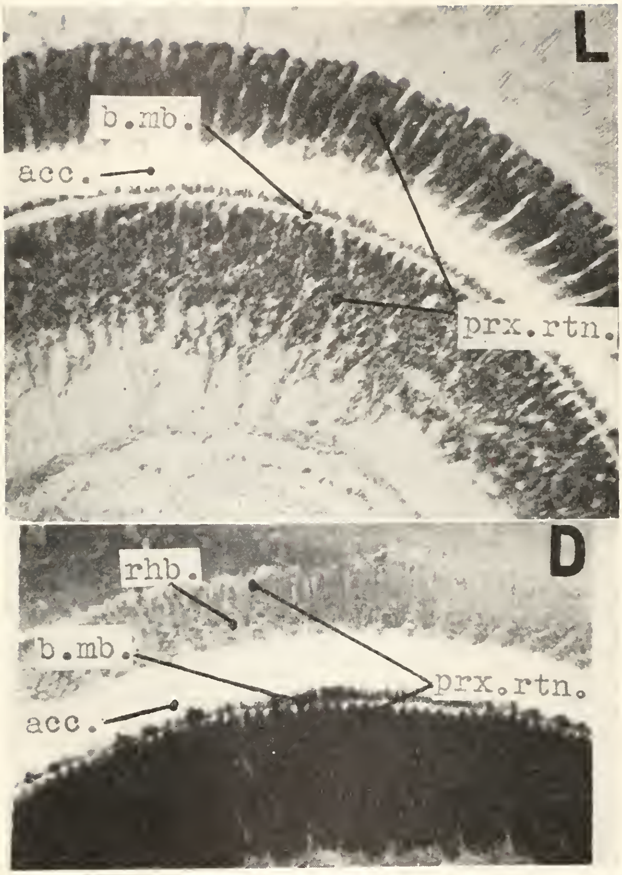
Oxygen and nitrogen were taken from commercial cylinders. Carbon dioxide was taken first from a Kipp generator, later from a commercial cylinder; no difference in results was detected.

Oxygen determinations were made by the Winkler method, modified after Kemmerer, Bovard, and Boorman (1923). Free carbon dioxide was determined by the method of Seyler (1894). The pH was determined colorimetrically by the use of the Clark and Lubs standard indicators bromthymol blue, bromcresol purple, and cresol red.

The temperature of the water was taken with each sample, and the variation was found to be within 3° C. for any given experiment.

The exact position of the proximal retinal pigment was recorded in every case, but for convenience in tabulation three general positions are used in this paper: (1) "Dark"—pigment not distal to the bases of the rhabdomes (Fig. 1, *D*; Plate 1, *D*); (2) "intermediate"—pigment extending along the rhabdomes but not distal to them; (3) "light"—pigment extending beyond the distal ends of the rhabdomes, *i.e.*,

PLATE I



Photographs of unstained sections of the compound eyes of *Cambarus virilis*, in the light-adapted (*L*) and dark-adapted (*D*) conditions. Only a part of each retina is shown, and only the proximal components of the ommatidia (cf. Fig. 1), *viz.*:

acc., accessory pigment layer; *b.mb.*, basement membrane; *prx.rtn.*, proximal retinal pigment cells, showing the nuclei at their distal ends, and extending proximally through the basement membrane; *rhb.*, rhabdomes, visible in (*D*) as the crenulated areas among the proximal retinal cells, but obscured in (*L*) by the retinal pigment. × 125.

nearly or quite to the tips of the proximal pigment cells (Fig. 1, *L*; Plate 1, *L*).

EXPERIMENTS

General Effect of Excess Carbon Dioxide

Preliminary experiments were performed in this laboratory by Mr. L. M. Schmidt. He did not determine the oxygen content or carbon dioxide content of the water, but he found that exposure to an excess of carbon dioxide (1) inhibited proximal migration of the proximal retinal pigment when light-adapted crayfish were placed in the dark and (2) brought about distal migration of this pigment in dark-adapted animals which were kept in darkness. His method was to bubble carbon dioxide (2 liters per hour) for six hours through water containing a number of crayfish, keeping other animals in running water as controls. All his animals were alive and were moving their swimmerets at the end of the six-hour period. Schmidt's results appear in Table I.

TABLE I

General effect of excess carbon dioxide on the position of the proximal retinal pigment —preliminary experiments.

	Number of eyes examined		
	Dark	Intermediate	Light
Controls—killed at end of experiment after 6 hours in darkness	12	2 *	0
Light-adapted animals, placed in darkness for 6 hours in dishes through which CO_2 bubbled at rate of 2 liters per hour	0	0	18
Controls—killed in darkness at beginning of exposure to CO_2	7	6 *	0
Dark-adapted animals, kept in darkness for 6 hours thereafter in dishes through which CO_2 bubbled at rate of 2 liters per hour	0	0	34

* The observed variation in pigment-position in the proximal cells of dark-adapted eyes was probably due to the fact that some of the controls were killed during the day, others at night. Bennitt (1932*b*) found evidence of diurnal variation in pigment position, even when the crayfish were in continuous darkness.

Relation between Position of Proximal Retinal Pigment, Carbon Dioxide Content, Oxygen Content, and Hydrogen Ion Concentration

Carbon dioxide was bubbled through about five inches of water in a 12 x 18 inch aquarium at the rate of 3 liters per hour. Eight or ten dark-adapted crayfish in the aquarium were prevented from rising to the surface by a layer of sheet cork. The apparatus was kept in darkness, and at intervals of one or two hours animals were killed and water samples were taken for determination of pH, oxygen content, and carbon dioxide content.

The experiments involved 42 animals (84 eyes); 21 animals (the "dark controls") were killed at the beginning of the exposure to carbon dioxide; the other 21 were kept in water through which carbon dioxide passed, and were in darkness throughout. The results appear in Table II, in which the figures for oxygen content, carbon dioxide content, and pH represent averages for the five experiments.

TABLE II

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Carbon dioxide bubbled through the water at the rate of 3 liters per hour. Animals in darkness throughout the experiment. (*—cf. foot-note, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	3.2	1.2	7.6	34	6 *	2 *
Continued in darkness; CO_2 for 2 hrs. at rate of 3 liters per hr.	0.8	45.0	6.0	0	6	12
Same conditions—4 hrs.	0.0	66.0	5.3	0	0	12
Same conditions—6 hrs.	0.0	124.0	5.3	0	0	12

TABLE III

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Carbon dioxide bubbled through the water at the rate of 6 liters per hour. Animals in darkness. (*—cf. foot-note, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	3.4	35.4	7.7	2	4 *	0
Continued in darkness; CO_2 for 30 min. at rate of 6 liters per hr.	0.2	137.5	5.4	2	4	0
Same conditions—60 min.	(water-sample lost)			4	2	0
Same conditions—90 min.	0.0	277.1	4.4	0	0	6

As the experiment progressed, the carbon dioxide content in each case increased while the pH and the oxygen content decreased. In an attempt to determine whether or not there was any quantitative relation between the rapidity of pigment migration and the rapidity of change of these physical values, carbon dioxide was next bubbled through the water at twice the former rate (*i.e.*, 6 liters per hour), and animals and water samples were removed every thirty minutes. The results appear in Table III.

All the animals in this experiment were light-adapted at the end of one and one-half hours. When carbon dioxide had been administered only half as rapidly (*i.e.*, 3 liters per hour), this condition was not attained until between three and four hours. The rapidity of migration of the proximal pigment appeared to vary in general with the rapidity of administration of carbon dioxide, though whether or not carbon dioxide itself was the active agent in the migration remained to be seen.

TABLE IV

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Nitrogen bubbled through the water at the rate of 2–3 liters per hour. Animals in darkness. (*—cf. footnote, Table I.)

	Oxygen	CO_2	pH	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls (beginning)	2.1	2.7	7.5	20	6 *	10 *
Continued in darkness; N_2 for 2 hrs. at rate of 2–3 liters per hr.	0.6	8.0	7.4	0	8	20
Same conditions—4 hrs.	0.3	7.7	7.4	2	2	26
Same conditions—6 hrs.	0.0	14.2	7.3	0	0	12
Dark controls (end)	—	—	—	18	12 *	6 *

Comparing now the physical conditions, shown in Tables II and III, which obtained when distal migration was complete in all the eyes, it appeared that while the oxygen content dropped to zero in both cases, there was little uniformity in the values for either carbon dioxide content or pH. The next step was to determine whether the pigmentary response was associated with (1) surplus carbon dioxide, (2) oxygen deficiency, or (3) increase in hydrogen ion concentration.

Effect of Oxygen Deficiency with Only a Slight Increase in Carbon Dioxide Content

Instead of carbon dioxide, nitrogen was bubbled through the water at the rate of 2 to 3 liters per hour. This permitted the free oxygen to be used up as in the preceding experiments without at the same time adding artificially a large amount of carbon dioxide to that already present. The procedure was as before except that "dark controls," kept in running water, were killed at both the beginning and the end of each exposure to nitrogen. The results of the six experiments are summarized in Table IV.

Since complete distal migration occurred in all animals killed when the CO_2-content was as low as 14.2 cc. per liter in the experiments with nitrogen, while it did not occur at much higher concentrations of carbon dioxide in previous experiments (cf. Tables II and III), it appeared that the concentration of carbon dioxide as such was not a factor affecting pigment migration in the proximal retinal cells except insofar as it might contribute to other physical changes. Moreover, since distal migration occurred in all cases at a pH as high as 7.3 in the experiments with nitrogen, while failing to occur at a pH as low as 6.0 in the earlier experiments (cf. Tables II and III), it would seem that the hydrogen ion concentration also was not a directly contributing factor. However, additional experiments were undertaken to settle this point.

TABLE V

Relation between position of the proximal pigment, oxygen content, carbon dioxide content, and pH. Water acidified with HCl. The pH-range represents readings taken at the beginning and end of each experiment.

	Oxygen	CO_2	pH range	Number of eyes examined		
				Dark	Intermediate	Light
	cc./liter	*cc./liter*				
Dark controls	1.7	1.5	7.6–7.6	12	0	0
Continued in darkness; acidified water for 2 hours	(no determinations)		5.2–5.6	4	2	0
Same conditions—4 hours	0.2	5.6	5.0–6.0	8	4	2
Same conditions—2 hours	3.4	44.0	3.9–4.8	2	8	16

Effect of Altered Hydrogen Ion Concentration

Tap-water was acidified with HCl, and dark-adapted animals were left in it in darkness for varying periods. The "dark controls," as before, were kept in running water. The results of these experiments are listed in Table V.

Thus hydrogen ion concentration appeared to become an active factor in promoting distal migration of the proximal retinal pigment only at acidities as high as pH 3–4, and even under these conditions the effect was by no means consistent, as is indicated by the 10 eyes which failed to become light-adapted. With one exception, however, the pH of the water in all previous experiments did not go below 5, and in all these cases distal migration had proceeded to completion. Hydrogen ion concentration, within the experimental range produced by the addition of carbon dioxide, did not affect appreciably the position of the pigment.

Discussion

It should be noted that the animals were neither dead nor even completely quiescent at the end of maximum exposures to carbon dioxide, nitrogen, or acidified water. In all cases the swimmerets were moving and in many cases the mouth-parts and other appendages as well. Check experiments showed that these animals could stand the most unfavorable conditions to which they were subjected in the experiments described in this paper, and would recover when replaced in running water.

The only one of the three factors tested which showed any consistent relation to migration of the proximal retinal pigment was oxygen content. As this decreased, the pigment began to move toward the distal position, even in darkness, and this migration was invariably completed when the oxygen tension had dropped to zero. If we are correct in supposing that in aquatic Crustacea there is a fair degree of correlation between the oxygen content of the water and the metabolic activity of the animal (as expressed by its oxygen consumption) it seems reasonable to believe that the pigmentary changes here observed are associated in some way with varying metabolic conditions. Evidence that the supposition is correct is afforded by Amberson, Mayerson, and Scott (1925). In a large number of experiments on the lobster (*Homarus*) they found a correlation between oxygen tension in the water and oxygen consumption by the animal so close as to warrant the statement that "at every instant the oxygen-consumption is directly proportional to the oxygen-tension in the sea-water at that instant" (1925, p. 175). They found an equally close correlation of the same sort in the polychaet *Nereis*, and preliminary experiments indicated a similar condition in the horse-shoe crab (*Limulus*), the blue crab (*Callinectes*), and the shrimp (*Palaemonetes*). *Palaemonetes* was able to keep its oxygen consumption constant until the oxygen content of the surrounding water had dropped to 50 per cent of saturation, but when less oxygen than this was present, its response was similar to that of the lobster.

Since distal migration of the proximal retinal pigment, though influenced primarily by the presence of light, is promoted also by low temperature, oxygen deficiency, anaesthesia, and death, it begins to look as though it might be promoted by any general factor which tends to retard metabolic activity. This idea was first presented by W. H.

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Cole (1922, p. 408) with reference to the distal movement of melanophore pigment in frog tadpoles—an activity analogous in many respects to distal movement of the proximal retinal pigment in crustaceans.

The rhythmical activity of the distal and proximal pigment cells of the crustacean retina under constant conditions of light or darkness (Welsh, 1930*a*; Bennitt, 1932*b*) is a condition which scarcely admits of interpretation except on the basis of daily changes within the animal's body. It is probable that such rhythmical activity as well may ultimately be explained on the basis of variations in metabolic activity.

SUMMARY

1. Overcrowding of crayfish in an aquarium often results in distal migration of the proximal retinal pigment while the animals remain in darkness.

2. Bubbling of carbon dioxide through the water surrounding the crayfish has the same effect.

3. When carbon dioxide is passed through the water at the rate of 3 liters per hour, distal migration of this pigment is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 66.0 cc. per liter, and pH 5.3.

4. When carbon dioxide is passed through the water at the rate of 6 liters per hour, distal migration is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 277.1 cc. per liter, and pH 4.4. The speed of distal migration varies in general with the speed of administration of carbon dioxide.

5. When, instead of carbon dioxide, nitrogen is passed through the water at the rate of 2–3 liters per hour, distal migration is complete at an oxygen content of 0.0 cc. per liter, a carbon dioxide content of 14.2 cc. per liter, and pH 7.3.

6. Acidification of the water with HCl, so long as the oxygen supply remains normal, produces no discernible effect on distal migration, except in the pH range 3.3–3.9, which is considerably below that involved in the preceding experiments.

7. The only factor shown to have a definite relation to distal migration in these experiments is oxygen deficiency. Distal migration is always complete when the oxygen content of the surrounding water reaches zero.

8. The various secondary factors promoting distal migration of the proximal retinal pigment—viz, low temperature, oxygen deficiency, anaesthesia, and death—all tend to retard metabolic activity. The evidence indicates that distal migration of this pigment, while influenced primarily by the presence of light, may secondarily be induced by agents which reduce the metabolic rate.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

III. The Applicability of Osmotic Laws to the Rate of Hemolysis in Hypotonic Solutions of Non-electrolytes

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I

Few subjects on the field of cellular physiology have received as much attention as that of osmotic hemolysis. Beginning with the observations of Hamburger (1886)—for a summary of the early literature, see Hamburger (1902)—there have appeared literally hundreds of papers dealing with this process under all conceivable conditions of health and disease, and of age, sex, species, and previous treatment of the blood. As has been pointed out in the preceding paper of this series (Jacobs and Parpart, 1931), much of this work is of very doubtful value because of the neglect of certain essential experimental precautions. Entirely apart from this defect, however, the work is disappointing in its almost complete failure to deal with the question of the rate at which such hemolytic processes occur. Unlike the published work upon other types of hemolysis (saponin, bile salts, specific sera, etc.), that upon osmotic hemolysis with few exceptions has had to do merely with the end state finally reached by the system. Information about this point, though possessing a limited practical value in medicine, is, on the whole, of comparatively little theoretical interest. So-called "fragility" studies—at least as ordinarily carried out—are concerned with the specific properties of the erythrocyte alone and they throw little light upon more fundamental problems of cell physiology. On the other hand, a study of the *rate* of osmotic hemolysis, which is closely associated with the rate of entrance of water into the cell, has obvious applications to many important general problems in the fields of osmosis, cell permeability, etc.

To all investigators who have tried in the usual way to measure the rate of osmotic hemolysis the reason for the almost complete neglect of this field in the past is clear. Osmotic hemolysis, when it goes to completion, as, for example, in distilled water or in very strongly hypotonic solutions, is such a rapid process—requiring for its entire course perhaps only a few seconds—that the ordinary methods of studying hemolytic phenomena cannot be successfully employed with it. On the other hand, when it is sufficiently slow to permit ready measurement, especially when the end-point is some partial degree of hemolysis, the results obtained are likely to be so variable and irregular, and at first sight so generally inexplicable, that most persons who have tried to work under these conditions have soon abandoned their attempts. The author has pointed out elsewhere (Jacobs, 1927, 1928, 1931; Jacobs and Parpart, 1931) that while irregularities in the behavior of the erythrocyte can be minimized by a strict standardization of the experimental procedure, they are in part inherent in the nature of the material itself and are therefore unavoidable. The difficulty, in brief, is that factors such as temperature, pH, etc. which might be expected to affect the rate at which a given equilibrium condition is attained, have an unusually strong tendency in the erythrocyte to change at the same time the position of the equilibrium itself. Under these conditions the results are, in general, too complicated for ready analysis; and the experimenter is of necessity driven back to the other horn of the dilemma where the difficulty is with the rapidity of the process and therefore with the method rather than with the material.

Fortunately, the simple method of the author (Jacobs, 1930) for the study of hemolysis proves to be adequate for a fairly accurate determination of times of hemolysis greater than approximately 1.2 seconds and permits experiments on the rate of the process to be made under conditions where disturbing equilibrium factors are of negligible importance, namely, in distilled water and in very strongly hypotonic solutions. The method therefore opens to experimental study an important field which has heretofore been almost wholly neglected. This general field will be dealt with in the present and in several succeeding papers.

A very fundamental question, which must first be decided before other work can be undertaken with profit, is how far the rate of hemolysis in hypotonic solutions may be considered to depend upon the rate of entrance of water into the erythrocyte in accordance with simple osmotic laws. In an earlier paper by the author (Jacobs, 1927) it was tentatively assumed that the erythrocyte behaves as a simple osmometer and gives up its hemoglobin to the surrounding solution when a certain critical hemolytic volume, V_h, is reached. On this assumption equations were derived for the calculation of permeability constants for water from data on the rate of osmotic hemolysis. It was, however, emphasized in another place (Jacobs, 1931) that osmotic hemolysis is in reality a fairly complicated process involving (*a*) the entrance into the cell of water, (*b*) the escape of hemoglobin, (*c*) the possible escape of salts and other osmotically active materials and (*d*) changes produced in various other ways in what is commonly loosely spoken of as the osmotic resistance of the cell. Only where factors *b*, *c* and *d* can be shown to be of negligible importance is it permissible to use the simple method of treating the subject previously employed; and a more critical examination of this point is therefore highly desirable.

A further need for such an examination is created by the recent work of Ponder and Saslow (1931), who have given reasons for doubting the applicability of simple osmotic laws to the erythrocyte because of the leakage from the cell during the course of at least certain types of experiments of osmotically active materials (factor *c* mentioned above). It must be admitted that in cases where such leakage occurs to any considerable extent a simple treatment of the problem is impossible. However, in view of the fact that Ponder and Saslow dealt primarily with final equilibria, arrived at in the course of a considerable time, it is by no means certain that in hemolytic experiments whose duration is only a few seconds such leakage as they have described would be a disturbing factor, though it is not impossible that it might. The question can be settled only by experiment, preferably by a comparison of the observed rates of osmotic hemolysis with those deduced according to the theory that the erythrocyte behaves as an ideal osmometer. Such a comparison will now be made.

II

The equations given in the earlier paper (Jacobs, 1927) for relating the time of hemolysis to the concentration of the medium are somewhat inconvenient because they employ the initial volume, V_0, and the hemolytic volume, V_h, of the cell. Though it has recently been shown by Ponder and Saslow (1931) that the idea of a hemolytic volume has actual experimental justification, there is, in general, a certain ambiguity in working with volumes. This is due to the fact that the volume that enters into osmotic equations is not the measured volume of the cell but rather that of the water which the cell contains. In some cases, *e.g.*, the cells of the plant *Tradescantia* (Höfler, 1917), this distinction is unimportant, but in the erythrocyte, which is loaded with hemoglobin to an extent of over 30 per cent by weight, it undoubtedly is—though, unfortunately, there is little agreement among different workers as to the actual magnitude of the true volume. For many purposes it is perhaps best to use the weight of the water in the cell as determined by chemical analysis (Van Slyke, Wu and McLean, 1923, etc.), but since under ordinary experimental conditions the weight of the water and what might be called the osmotically effective volume of the cell, *i.e.*, that part of the total volume which takes part in osmotic changes, are approximately related in a very simple manner, it is perhaps permissible for more ready comparison with other published work in this field to retain for the present purposes the older type of osmotic equations involving volumes and concentrations.

Assuming with Hill (1930) that the water within the cell is almost entirely "free," *i.e.*, capable of taking part in osmotic equilibria, or at least that there are no marked changes during the course of the experiment in the degree of "binding" of water by cell constituents, we have the relation:

$$cV = c_0 V_0,$$

where c_0 and V_0 are the initial osmolar concentration and osmotically effective volume of the cell, respectively, and c and V are any other corresponding pair of these variables. The hemolytic volume, V_h, used in previous discussions may therefore be expressed in terms of constants and of the more convenient hemolytic concentration:

$$V_h = V_0 \frac{c_0}{c_h}.$$

Making certain necessary and probably well justified simplifying assumptions as to the nature of the diffusion of water across the membrane of the erythrocyte (see in this connection Northrop, 1927; Lucké, Hartline and McCutcheon, 1931; Jacobs and Stewart, 1932), it may be predicted that the rate at which it will enter the cell, *i.e.*, the rate of increase of the cell volume, will at any given instant be proportional to the difference in the osmotic pressures, and therefore to that of the concentrations of the internal and external solutions, and to the extent of surface of the cell.

$$\frac{dV}{dt} = kA(c - C). \tag{1}$$

The external concentration, C, may be considered to be constant since the volume of the surrounding solution is very large as compared with that of the suspended cells (approximately 1000 : 1 in these experiments). Since

$$V = V_0 \frac{c_0}{c},$$

we may write equation (1) in the form:

$$-\frac{c_0 V_0}{c^2} \frac{dc}{dt} = kA(c - C). \tag{2}$$

Fortunately in the erythrocyte, because of its peculiar biconcave shape, a considerable degree of increase in volume is possible without any change in surface. No great error will result, therefore, if A be treated as if it were constant, and this simplifying assumption permits equation (2) to be integrated at once after separating the variables; that is:

$$kAt = -c_0V_0\int\frac{dc}{c^2(c-C)}\cdot$$

Remembering that when $t = 0$, $c = c_0$, the initial isotonic concentration for blood, we finally obtain:

$$kAt = \frac{c_0V_0}{C^2}\ln\frac{cc_0 - cC}{cc_0 - c_0C} + \frac{c_0V_0}{C}\left(\frac{1}{c_0} - \frac{1}{c}\right)\cdot \tag{3}$$

For the special case where the cells swell in distilled water, and C in equation (2) is therefore equal to zero, a simpler equation results, namely,

$$kAt = \frac{c_0V_0}{2}\left(\frac{1}{c^2} - \frac{1}{c_0^2}\right)\cdot \tag{4}$$

As has already been stated, V_0, the initial osmotically effective volume of the cell, is not exactly known but is, in any case, a constant. For our present purposes, therefore, V_0 and A, which has also been treated as a constant, may be incorporated with the true permeability constant, k, to give a quantity, k', whose constancy over a range of concentrations would equally well furnish a proof of the correctness of equations (3) and (4). Since we wish to use the equations only for the point at which hemolysis occurs, namely t_h, c_h, we substitute these particular values for t and c, respectively, and also for convenience represent the ratio c_0/c_h by R, giving finally:

$$k't_h = \frac{c_0}{C^2}\ln\frac{c_0 - C}{c_0 - RC} - \frac{R-1}{C} \tag{5}$$

and

$$k't_h = \frac{1}{2c_0}(R^2 - 1). \tag{6}$$

If, therefore, the erythrocyte behaves as a simple osmometer, equations (5) and (6) should yield the same value of k' for all values of C including the value of zero when distilled water is employed.

III

To test the applicability of the theoretical equations derived in the preceding section to osmotic hemolysis, experiments were performed upon erythrocytes by the method mentioned above (Jacobs, 1930). In the present paper only a single one of the earlier experiments with saccharose solutions will be described in detail; but it may be mentioned that essentially the same results have been obtained in a considerable number of other experiments both with this substance and with dextrose. Some of the later confirmatory experiments were performed by A. K. Parpart, whose careful assistance is gratefully acknowledged.

The blood used for the experiment here described was that of the ox, defibrinated immediately after its collection. Because of certain abnormalities that seem to develop when erythrocytes stand for some time in contact with protein-free salt solutions (Kerr, 1929), the cells were not "washed" but were kept in the approximately normal surroundings furnished by their own serum up to the instant of exposure to the hemolytic solutions. It should be noted that previous washing in isotonic non-electrolyte solutions is also contraindicated by the tendency shown by erythrocytes to become agglutinated in such solutions. Though the procedure that was of necessity followed resulted in the introduction into the non-electrolyte solutions of slight traces of electrolytes and of proteins, these were small since the dilution of the blood employed was approximately 500 : 1 and that of the serum therefore of the order of 1000 : 1, giving a final concentration of electrolytes in the vicinity of M/6000.

TABLE I

Times of hemolysis of ox erythrocyte in water and in saccharose solutions. R is assumed to have a value of 2.1.

Concentration	Freezing point depression	Observed time of hemolysis in seconds	k'
0.00	0.00	1.40	2.10
0.10	0.0186	1.53	2.02
0.02	0.0373	1.60	2.05
0.04	0.0747	1.65	2.26
0.06	0.1123	1.73	2.49
0.08	0.1501	1.90	2.70
0.10	0.1880	2.15	3.00
0.12	0.2261	2.50	3.55
0.13	0.2452	2.75	4.12
0.14	0.2643	3.53	4.77
0.145	0.2739	7.33	3.88

With each solution four determinations of the time of hemolysis were made to the nearest tenth of a second. These figures have been averaged to give the times listed in the third column of Table I. As a rule, the individual observations in each group of four varied by only one or two-tenths of a second. Only with the highest concentrations employed, where the complicating factors previously mentioned are present, did the observations fail to show a high degree of reproducibility. The concentrations of the solutions employed are given in the first column of the table, but for purposes of calculation the freezing point depressions were used as being more nearly proportional to the osmotic pressures than the concentrations. These were calculated by the empirical equation:

$$\Delta = 1.86C - 0.2C^2,$$

which fits very closely the data given in the "International Critical Tables" for saccharose over the range of concentrations employed.

The critical concentration for 75 per cent hemolysis, which is in practice a convenient end-point to use, was directly determined as 0.148M with a calculated freezing point depression of 0.280°. Since the freezing point depression for ox serum is in the vicinity of 0.58° (Hamburger, 1902) R, the ratio of c_0 to c_h may be taken as approximately 2.1.

From these data and from the observed rates of hemolysis, Table I has been prepared. An inspection of the figures in the first and third columns of this table, or, better, of the positions of the solid circles in Fig. 1, shows the relation between the observed times of hemolysis and the concentrations of the external solutions. It will be noted that an increase in concentration from zero (distilled water) to 0.12M or 0.13M has only a relatively slight retarding effect. The retardation then increases at a rapid rate and at a concentration of 0.148M reaches infinity. This type of curve, characterized by the relative suddenness of its final rise, has always been obtained with non-electrolytes, but, as will be shown in a later paper, not with electrolytes. How far does it agree with the theoretical predictions made by the use of equations (5) and (6)?

The answer to this question may be presented in two ways. In the first place, in the last column of Table I are found the calculated values of k' for the various concentrations employed. It will be noted that while there is good agreement between the values for water and for 0.01M and 0.02M saccharose, this agreement soon disappears and the last values of k' are about 100 per cent greater than the first ones. This lack of agreement is almost certainly not the result of fortuitous errors of observation, since the drift from smaller to larger values of k' is a very regular one. Evidently the predictions made from the theoretical equations depart rather widely from the observed data.

The same thing is shown still more clearly in Fig. 1 where the curve labeled 2.1 represents the times at which hemolysis ought theoretically to occur if the first few values of k' applied throughout the concentration range instead of increasing as they do with increasing concentration. It will again be noted that the agreement between theory and observation is very poor. More specifically, the observed values increase much too slowly until the highest concentrations are reached, and then they tend to increase very rapidly. It would appear, therefore, either that the rate of hemolysis is not governed in any very simple manner by osmotic laws or that some additional factor of importance has been overlooked in making the calculations.

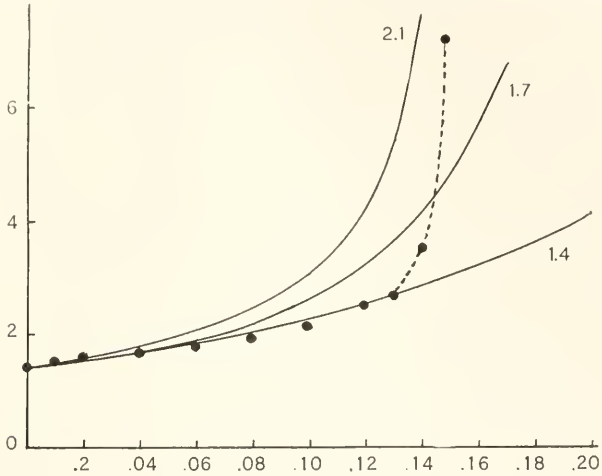

FIG. 1. Observed times for hemolysis of ox erythrocytes in distilled water and in saccharose solutions of different concentrations represented by the solid circles. Calculated times for different values of R represented by the curves. Ordinates represent times; abscissae, concentrations in mols per liter.

IV

A careful examination of the data suggests that the reason for the discrepancy between observation and theory lies not so much in the inapplicability of simple osmotic laws to the hemolytic process as in a serious error in obtaining experimentally the value of the critical hemolytic concentration, c_h. This error, in turn, is responsible for one in the value of R which appears in equations (5) and (6). An inspection of these equations, or even better, the actual substitution in them of several different values of R, all other figures remaining the same, shows that the effect of this constant on the calculated values of k' is very great and that even a small error in its determination must have serious effects. Now it might seem that of all the values entering into the calculations that of c_h, and consequently also that of R, are the most reliable, since the concentration of the solution in which a given degree of hemolysis—for example, 75 per cent—is finally reached can be determined by direct observation with a very high degree of accuracy. But before using for purposes of calculation values of c_h and of R as so determined account must be taken of a very suspicious circumstance, namely, that the osmotic pressure of the solution of sucrose which ultimately gives 75 per cent hemolysis is very different from those of diluted serum or of NaCl having the same hemolytic effect.

For example, in four separate experiments to be described in a later paper, it was found that the unbuffered solutions of NaCl in which 75 per cent hemolysis was ultimately attained with a dilution of blood of approximately 1 : 500 had concentrations of 0.097M, 0.093M, 0.104M and 0.098M, with an average value of 0.098M. The freezing point depression of such a solution is approximately 0.340°, giving a value of R of 1.7 instead of 2.1 as determined above. In other words, erythrocytes in solutions of saccharose and, as may readily be shown, in solutions of other non-electrolytes also, have a higher resistance than in solutions of electrolytes or in diluted serum. This effect has been noted by many workers and has been variously explained. Leaving undetermined for the present the exact mechanism by which it is produced we may accept it as a known fact and consider some of its possible consequences.

When an erythrocyte is placed in a non-electrolyte solution it possesses certain osmotic properties which are changed by exposure to its new surroundings. The critical hemolytic concentration as actually determined in such solutions is, therefore, obviously that of a cell whose properties have been modified and not that which the unaltered erythrocyte ought theoretically to show. But suppose, as seems likely, that this change is not an instantaneous one, but requires an appreciable time, say, 10 seconds, for its completion. Evidently under these conditions very rapid hemolytic processes requiring only two or three seconds would be finished before much change in the erythrocyte could occur; on the other hand, if the duration of the hemolytic process were 15 or 20 seconds, it would be sufficiently slow to permit the change—in this case an increase in the resistance of the erythrocyte—to take place. Indeed, a point would rather suddenly be reached where the increased resistance developed by the erythrocyte would be sufficient to prevent completely the hemolysis that would otherwise occur; and at this point there would be a sudden rise of the time-of-hemolysis curve to infinity.

As has been noted above, the curve expressing the observed relation between concentration and time of hemolysis shows as its most striking peculiarity a sudden rise as the highest concentrations are approached. By far the simplest explanation of this peculiarity—indeed the only one that has so far occurred to the author—is that the increased resistance which is known to be brought about in non-electrolyte solutions does not develop instantly but requires for its appearance several seconds—almost certainly more than two or three. If this rather plausible view be accepted, then it is obviously erroneous to use the observed value of c_h in calculations involving water and very dilute solutions in which hemolysis occurs in from 1.4 to 2 or 3 seconds, *i.e.*, before the original osmotic properties of the erythrocyte have been greatly altered. Some other higher value of c_h would evidently govern the behavior of the erythrocyte in such media. Let us assume as a first approximation that the true value of c_h is that determined by the use of NaCl rather than by sugar solutions. From the figures given above, the new value of R would be in the vicinity of 1.7 (*i.e.*, $0.58 \div 0.340$). Using this figure as preferable to the old one of 2.1 there have been calculated the new values of k' in column 2 of Table II.

TABLE II

Values of k' calculated from the data of Table I, assuming $R = 1.7$ and $R = 1.4$.

Concentration	$R^{k'} = 1.7$	$R^{k'} = 1.4$	Concentration	$R^{k'} = 1.7$	$R^{k'} = 1.4$
0.00	1.16	0.59	0.08	1.34	0.63
0.01	1.11	0.51	0.10	1.39	0.64
0.02	1.12	0.56	0.12	1.46	0.63
0.04	1.20	0.59	0.13	1.48	0.62
0.06	1.29	0.62	0.14	1.32	0.53

It is immediately apparent on examination of this table (see also the curve labeled 1.7 in Fig. 1) that the agreement between observation and theory, as indicated by the relative constancy of k', is now considerably better than before, though there is still a slow drift in the constant, which can scarcely be accounted for by experimental errors. It is to be noted, however, that the critical concentration as inferred from NaCl experiments is itself probably too low. According to Ponder and Saslow (1931), in experiments whose duration is of the order of magnitude of the time used to determine this figure (1 hour), there is a change, interpreted by them as due to an escape of salts from the erythrocyte, which is sufficient to influence the cell-volume and which would undoubtedly render hemolysis by hypotonic solutions more difficult than otherwise. If this conclusion be accepted, then the observed critical concentration, c_h, is still too low and the assumed value of R of 1.7 is too high.

Though it is impossible at present to be certain what further correction is justified, it may be of interest to assume for the unaltered cell a value of R of 1.4. Calculations made by using this figure are given in column 3 of Table II. It will be noted that the value of k' is now almost constant, indicating an agreement of theory and observation, up to a concentration of 0.13M. This agreement is even more strikingly shown in Fig. 1 where the curve labeled 1.4 has been calculated for this value of R by means of equations (5) and (6), starting with 1.40 seconds as the time required for hemolysis in distilled water. An even better agreement could be obtained by taking a slightly lower value of R; but, in view of the simplifying assumptions used in deriving the equations, it is questionable whether the almost perfect fit that could be secured in this way has any very great significance. The important thing is that by assigning a not-improbable value to c_h (the theoretical hemolytic concentration for *the unaltered cell*), the behavior of the erythrocyte over a wide range of concentrations shows a good agreement with simple osmotic laws, and its deviation from these laws at very high concentrations can be plausibly accounted for.

V

Up to this point the increased osmotic resistance of the erythrocyte in solutions of non-electrolytes has been accepted merely as an observed fact with no attempt at an explanation. Though for present purposes it is not strictly necessary that the cause of this peculiarity of the erythrocyte should be known, it may be noted that there are not lacking a number of more or less plausible explanations which because of their general theoretical interest may now be briefly considered.

The first explanation is that non-electrolytes actually have a toughening and strengthening effect upon the cell membrane which renders the erythrocyte less susceptible to hemolysis (see for example Rhode, 1923). Although a solidifying effect of non-electrolytes upon certain gels and upon both plant and animal cells has frequently been observed (for literature upon this subject see Höber and Memmesheimer, 1923; and Höber, 1926), it seems very unlikely that this explanation is capable of accounting for such a remarkable increase in the osmotic resistance of the erythrocyte as is known to occur. In the first place, the membrane of this cell is so delicate that it probably offers little opposition under any conditions to volume changes (for some of the evidence see Jacobs, 1931). Even the much better developed membrane of the egg of *Arbacia* seems to be capable of resisting only very feebly osmotic volume changes (Lucké and McCutcheon, 1932; Harvey, 1931; Cole, 1932). It is almost inconceivable that the membrane of the erythrocyte could be so strengthened in the absence of electrolytes as to support an excess osmotic pressure of over an atmosphere (*i.e.*, the difference in the osmotic pressures of solutions of NaCl and of saccharose, which just permit 75 per cent hemolysis to occur). Furthermore, according to Ponder and Saslow (1931) osmotic hemolysis does not necessarily involve any very appreciable stretching of the cell membrane. It seems unlikely, therefore, that a direct mechanical effect of this sort on the cell is chiefly involved.

A second possibility is somewhat more plausible. It is that in a hypotonic non-electrolyte solution there is a sufficient leakage of electrolytes from the interior of the cell to lower the internal osmotic pressure and so to reduce the amount of swelling that would otherwise occur. This explanation of the increased osmotic resistance of the erythrocyte in non-electrolyte solutions has been accepted, among others, by Bang (1909) and by Ponder and Saslow (1931). The last named authors suppose "that leakage is greater in glucose than in NaCl and that this accounts for the critical volume being reached in a solution of glucose which is more hypotonic than one of NaCl." They cite in support of this view the direct chemical evidence obtained by Kerr (1929) that in solutions deficient in blood proteins there may be an escape of potassium from and an entrance of sodium into the cell. Bang (1909) also gives references to earlier work indicating a passage of electrolytes from the erythrocyte into non-electrolyte solutions, while Joel (1915) has studied this process and the influence upon it of narcotics, by an electrical conductivity method. That electrolytes may escape from the erythrocyte into non-electrolyte solutions may therefore be regarded as a well-established fact.

It is very questionable, however, whether such an escape of electrolytes, which is probably associated with a loss of the normal permeability of the cell to cations, is capable of accounting for the very rapid rise of osmotic resistance that occurs in the present experiments. From the data shown graphically in Fig. 1 it would seem that a marked increase in resistance in non-electrolyte solutions must occur in less than five seconds, while even under the conditions of their experiments, Ponder and Saslow (1931) state that "the equilibrium volumes are attained within a minute and are maintained for hours." Since the diffusion of cations reported by Kerr (1929) is a process that seems to extend over hours, while the rise in conductivity studied by Joel went on gradually and steadily throughout experiments also lasting up to several hours, it would seem that some factor other than an outward leakage of salts (*i.e.*, of both anions and cations) is involved in the case of very rapid changes. The factor that immediately suggests itself is a new ionic equilibrium of some sort, attained primarily by the movement of anions such as is known to occur readily in normal erythrocytes. How far the results of Ponder and Saslow with electrolyte solutions can be so explained cannot at present be stated with certainty; but, at all events, it seems likely that the extremely rapid increase in the osmotic resistance of erythrocytes that takes place in non-electrolyte solutions is to be accounted for in this way.

This view is supported by the work of Netter (1928), who has pointed out that theoretically ionic exchanges should by no means be absent between erythrocytes and a surrounding isotonic solution of a non-electrolyte, but that anions from the erythrocytes would tend to be exchanged for OH' ions from the aqueous solution in such a way as to make the interior of the cells more alkaline. This principle has been put to practical use by Bruch and Netter (1930) in obtaining various desired relations between external and internal pH values. Now it is known, especially from the work of Warburg (1922) and of Van Slyke, Wu and McLean (1923), that a change in reaction within the erythrocyte has important osmotic consequences. The osmotic pressure of a given amount of base bound by hemoglobin is considerably lower than that of the same amount of base bound by, for example, carbonic acid. If the compound of base with hemoglobin be represented as B_nHb, the osmotic pressure of this compound when completely dissociated would be to that of the same amount of base combined with carbonic acid as $(n + 1) : 2n$. Anything, therefore, which causes a shift of base from hemoglobin to carbonic acid should increase the internal osmotic pressure of the cell and cause the latter to swell; anything that causes a shift in the reverse direction should have the opposite effect. Since the new ionic equilibrium attained in non-electrolyte solutions is obviously of the latter nature, it should, without any escape of salts as such, result in a lowering of the internal osmotic pressure, and so raise the resistance of the cell to hemolysis by hypotonic solutions.

If this view of the mechanism of the increase in the osmotic resistance of the erythrocyte in non-electrolyte solutions be correct, then it ought to be possible even in solutions of electrolytes to produce the same characteristically sudden rise in the time-of-hemolysis: concentration curves described above by slightly increasing the alkalinity of the medium with a trace of $NaOH$ or NH_4OH. This is, in fact, the case, as will be shown in a forthcoming paper by the author in collaboration with A. K. Parpart. For this reason, as well as for the others mentioned above, it seems likely that while a leakage of salts (*i.e.*, of both anions and cations) is by no means excluded as a possible factor of importance in experiments of longer duration, the factor chiefly concerned in producing increased resistance under the conditions of these experiments is an entirely normal shift of anions alone, which because of certain peculiarities of hemoglobin, is secondarily responsible for a change in the internal osmotic pressure of the cell.

VI

It is a matter of some interest to determine the value of the true permeability constant, k, of equations (1), (3), and (4). This is a measure of the amount of water that would cross the membrane of the erythrocyte through unit area in unit time with unit difference in osmotic pressure between the cell and the surrounding solution. Such a constant would be useful for comparing the permeability to water of different cells, or of the same cell under different conditions (see Jacobs 1927, 1931).

The true permeability constant, k, is found from k' of equations (5) and (6), by multiplying by V_0, the initial osmotically effective volume of the cell, which may be measured in cubic micra, and dividing by A, the area of the cell, which is conveniently expressed in square micra. Unfortunately, both because of the small size of the erythrocyte and its peculiar shape, it is difficult to measure its surface with very great accuracy. This difficulty is well illustrated by the fact that the estimates made by different investigators for the erythrocytes of the same species of mammals may differ by as much as 50 per cent or more. Furthermore, even though the total volume of the cell can be fairly accurately determined by several methods (Ponder and Saslow, 1930) there has in the past been much uncertainty in calculating from it the osmotically effective volume, though it seems likely that this is in reality not very different from that of the total water contained in the cell (Hill, 1930). Finally, there is the serious difficulty, mentioned above, that because of uncertainty as to the value of the theoretical critical hemolytic concentration for the unaltered erythrocyte the value of k' itself is subject to a considerable error. It is evident, therefore, that the most that can be expected at present is to obtain the order of magnitude of the true permeability constant; but even this would be of considerable value.

For the ox erythrocytes chiefly used in these experiments we may take as a first approximation a value of R of 1.7. This value is obtained from the observed hemolytic concentration of NaCl rather than from that of saccharose, since, as has been mentioned above, the normal osmotic properties of the erythrocyte are very quickly changed in non-electrolyte solutions. Indeed, even the value selected is probably somewhat too high, but in the absence of more complete information it is a convenient one to use.

As to the necessary constants for the cell itself, the few published estimates are not in very good agreement. Probably the best available value for the volume of the ox erythrocyte is that given by Ponder and Saslow (1930) of 44 cubic micra. No estimates of surfaces are given in this paper, but in an earlier publication Ponder (1924) has given 37 cubic micra and 69 square micra as the values of the volume and surface, respectively, of the erythrocyte of the calf. If it be assumed that the shape of the somewhat larger cell is exactly the same as that of the smaller one, then its surface would be $69 \times \left(\frac{44}{37}\right)^{2/3}$ or 77 square micra, and this value will here be used.

In the absence of accurate chemical analyses of ox erythrocytes, it may tentatively, though perhaps somewhat questionably, be assumed that they contain the same percentage of water as those of man and that all of this water is "free." Taking an average of the figures given by Henderson (1928) for cc. of water in 1 liter of cells for three normal human individuals, and applying the same percentage to the ox erythrocyte whose total value is 44 cubic micra, we have for the initial effective osmotic volume, V_0, 0.69×44 or approximately 30 cubic micra. Remembering that the time for 75 per cent hemolysis in water is 1.4 seconds, we have all the data necessary to calculate from equation (6) the value of k.

$$k = 0.08 \times \frac{1}{1.4} \times \frac{1}{1.16} \times \frac{30}{77} \times [(1.7)^2 - 1] = 0.036.$$

The factor 0.08 has been introduced to change freezing point depressions in degrees centigrade into osmotic pressures in atmospheres. Expressed in words, the value of k so obtained means that with a difference in osmotic pressure of one atmosphere between the cell and its surroundings water should theoretically pass through each square micron of the cell surface at the rate of 0.042 cubic micra per second or of 2.2 cubic micra per minute.

In an earlier paper (Jacobs, 1927) the value of k was estimated to be of the order of 3.0 for human erythrocytes when the unit of time was taken as one minute. Since the details of the calculation were not given at that time, it may be worth while to present here an additional typical set of figures, emphasizing at the same time the fact that only the general order of magnitude of the results obtained from them is significant. In this particular case c_h in terms of freezing point depressions was found to be 0.232, while c_0 was taken as 0.56, giving a value of R of 2.4. The observed time of hemolysis was 2.4 seconds. Following Emmons (1927) the volume of the human erythrocyte may be taken as 88 cubic micra (of which 0.69 × 88 or 61 cubic micra represents the true osmotic volume) and the surface as 145 square micra. We have, therefore:

$$k = 0.08 \times \frac{1}{2.4} \times \frac{1}{1.12} \times \frac{61}{145} \times [(2.4)^2 - 1] = 0.060$$

or, if the unit of time be taken as the minute, 60 times this value or 3.6.

In view of the large unavoidable errors in these calculations, due especially to uncertainty as to the exact value of R, it is questionable whether this apparently greater permeability to water of the human erythrocyte as compared with that of the ox is significant. In any case, the difference in the permeabilities of the two kinds of erythrocytes to water is far less than is that to glycerol (Jacobs, 1927, 1931) or to erythritol and to certain ions (Mond and Gertz, 1929).

SUMMARY

1. Equations are derived for predicting the relation between the time required for osmotic hemolysis and the concentration of a surrounding hypotonic medium.

2. It is shown that when allowance is made for certain known peculiarities of the erythrocyte the rate of hemolysis is, on the whole, in fairly good agreement with osmotic laws.

3. Reasons are given for believing that the increased osmotic resistance of the erythrocyte that develops within a few seconds in solutions of non-electrolytes is not caused by a leakage of salts from the cell but rather by a changed ionic equilibrium in which the normal impermeability of the cell to cations need not be lost.

4. Rough quantitative estimates are made of the permeability of the erythrocytes of the ox and of man to water.

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LOCOMOTOR ORGANS OF ECHINARACHNIUS PARMA

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The method of locomotion in the sand-dollar, *Echinarachnius parma* (Lam.), is not clearly understood. MacBride in the Cambridge Natural History (1906, p. 548) states that "The locomotor tube-feet (in *Echinarachnius*) are very small and feeble compared with those of *Echinus esculentus*, but this is comprehensible when it is recollected how little resistance the yielding sand would offer to the pull of a powerful tube-foot like that of the Regular Urchins, for in order to move the creature through the sand a multitude of feeble pulls distributed all over its surface is necessary, and the locomotor tube-feet are exactly fitted, both as to size and number, for this object." It is quite plain from this quotation that MacBride is of opinion that locomotion in the sand-dollar is accomplished by the tube-feet. In conversation with a well-known specialist in this group of animals, the senior author was informed that *Echinarachnius* possesses no tube-feet at all excepting those that are modified for gills. When experts on the echinoderms differ so widely on a simple question of fact the obvious step is to reinvestigate the subject. The present studies were carried out at the Marine Biological Laboratory, Woods Hole, where an abundance of living specimens of *Echinarachnius* was available.

The locomotion of the sand-dollar has already been reported upon in an earlier paper by the senior author (Parker, 1927). In this publication, it was shown that *Echinarachnius* exhibits two types of locomotion, rotational and rectilinear. In the second type the sand-dollar creeps forward on an axis corresponding to that of its structure. This axis is represented by a straight line passing through the animal and intercepting mouth and anus. Although the animal is in general radially symmetrical, this axis divides it into right and left halves that are structurally significant in its locomotion. In addition to its rectilinear and rotational movements, the sand-dollar can right itself after having been thrown on its back. Such righting reactions also call for an appropriate locomotor mechanism. The question to be discussed in these pages is what structures are concerned with these several types of locomotion. Three classes of organs may be suspected of having to do with such activities. They are the integumentary cilia, the tube-feet (if present), and the spines.

1. *Integumentary Cilia.*—Integumentary cilia have been identified with certainty only on the spines. In *Echinarachnius* the spines are of two kinds, long and short. These two kinds occur on both the oral and the aboral surfaces. The short spines are provided with rounded tops and their cilia occur not only on the tops but also to some extent on their sides. In the long spines the cilia are present as elongated bands on the sides of the spines from the base to near the top. The ends of the long spines are devoid of cilia. It is questionable if any cilia occur on the general surface of the integument, though it is possible that they may be found in the ambulacral grooves. Gislén (1924, p. 247) was unable to demonstrate to his satisfaction the presence of cilia in the ambulacral grooves of the allied *Arachnoides* and *Astriclypeus.*

The direction in which these cilia beat can be determined by the flow of carmine particles discharged on the surface of the sand-dollar. On the aboral face, the ciliary currents flow from the center of the test outward toward the periphery on essentially radial lines anteriorly and posteriorly, but on somewhat curved lines right and left from the axis of locomotion. In these lateral regions the direction of flow is outward and backward. On the oral surface the ciliary currents are all from anterior to posterior, no part of the surface exhibiting currents which run toward the anterior edge. In all cases both oral and aboral, these currents are so weak that they are incapable of moving even small sand particles that happen to fall among them. There is therefore no reason to suppose that the ciliary currents are concerned with locomotion. They function in all probability in the respiratory exchange of water next the animal's body and probably remove from the skin accumulations of very light silt and other dirt.

2. *Ambulacral Feet.*—In a living *Echinarachnius* a continuous fringe of active ambulacral feet can be seen around the whole edge of the test where they are to be observed in incessant activity between the numerous spines. When fully extended, they are two to three times the length of the longer spines. In contraction they draw back close to the general surface of the animal. Each foot is provided with a well-developed terminal sucker deep pink in color. Ambulacral feet essentially like those along the edge may be identified on both the oral and aboral surfaces. Here in consequence of their deep color and characteristic arrangement they give rise to a pattern which in the living animal is often of striking appearance and symmetry. On the oral surface (Fig. 1) they extend as five somewhat irregular bands along the lines of the ambulacral grooves. As these bands approach the edge of the test they widen out till on the very edge they unite to form the continuous fringe already mentioned. On the aboral surface

(Fig. 2) five well-defined double ambulacral bands of tube-feet extend from near the center of the test to its edge. Each double band passes between the two halves of a respiratory rosette. A few tube-feet are often to be seen among the gills on the outer edges of the rosettes. As the double bands approach the periphery of the test they are supplemented on each side by double series of three or more large spots, containing many tube-feet. The double bands and the supplemental spots as they approach the margin expand to such a degree that they form the continuous edging of tube-feet already described. In general the tube-feet are especially numerous and long on the edge of the test, and fewer and shorter toward the center. The shortest and least active feet occur in the ambulacral grooves. Except in the regions previously noted we have found no ambulacral feet in *Echinarachnius.*

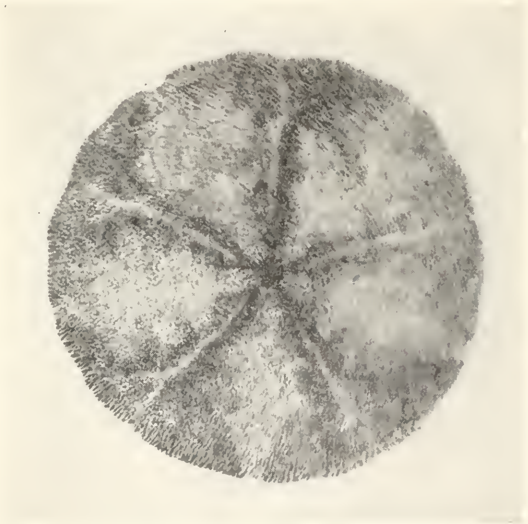

FIG. 1. Oral view of a living *Echinarachnius parma.* The dark areas beginning at the mouth and following the ambulacral grooves to the edge of the test near which they expand broadly and somewhat irregularly are the areas covered with ambulacral feet.

When a sand-dollar moves forward on the sand in process of burrowing, the tube-feet of the anterior edge pull the grains of sand in toward the center of the animal, particularly over the anterior half of the periphery. The action of the feet is not vigorous nor well coördinated. Those along the advancing edge are most active and succeed in piling the sand into a low mound on the front of the animal. In moving forward, the animal pushes itself under this mound and the tube-feet help thus to cover the aboral surface. It was difficult to determine the direction of pull of the feet in the middle of the oral side. When a sand-dollar was placed in a glass vessel and studied from below by means of a low-power microscope, the feet were seen to move less regularly than those along the edge of the test. In order to observe the movements of such feet, it was necessary to reduce the sand on the bottom of the glass to a very thin layer. This was unfavorable for the movements of the animal and may have been the occasion of the irregular movements seen under these circumstances. Possibly in deep sand the more centrally located feet would have shown a very different type of movement. The pulls of the feet on the posterior portion of the test were toward the mouth, but they were feeble and less coördinated than those of the anterior edge.

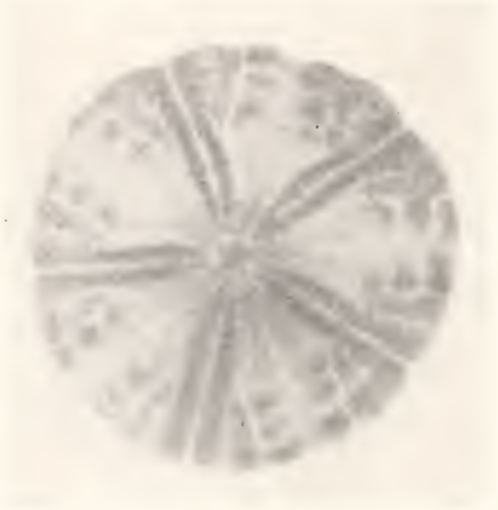

FIG. 2. Aboral view of a living *Echinarachnius parma*. The five pairs of radial dark bands and the marginal and submarginal dark spots are the areas covered with ambulacral feet.

There is thus no doubt of the presence of tube-feet on *Echinarachnius*, but it is improbable that the forward locomotion of this animal is in any essential way dependent upon them. In this respect our results fail to support the opinion already quoted from MacBride. The only part of *Echinarachnius* in which the tube-feet exhibit well coördinated and vigorous activity is along the anterior edge of the test, but even here their number and distribution is such that they cannot be regarded as more than subsidiary organs in the locomotion of this animal.

3. *Spines.*—The test of *Echinarachnius* is covered thickly with two kinds of spines, long and short. The only parts of the surface that lack spines are the troughs of the ambulacral grooves on the oral surface. The spines are longest near the anterior oral edge and diminish somewhat in size as one passes posteriorly from this region. As one approaches the posterior edge larger spines are again met with, but these posterior spines never reach in size those of the anterior margin. On the aboral surface the spines for the most part point toward the margin of the test, hence radially. On the oral surface the arrangement is less simple, but on both surfaces the spines exhibit a bilateral distribution in relation to the axis of locomotion. This arrangement of the spines has already been figured (Parker, 1927, Fig. 1). When a sand-dollar is placed on submerged sand, it will push forward into the sand until in 10 to 20 minutes it has covered itself completely. This operation involves forward locomotion through about ten centimeters of distance. After the sand-dollar has become covered it usually progresses less rapidly and may in fact stop moving altogether. As the animal moves forward in burying itself, the sand, as already stated, piles up over the anterior edge and gradually spreads over the aboral surface to the complete disappearance of the animal. This is due particularly to the forward movement of the animal as a whole and partly to the pull of the ambulacral feet. When sand is poured on the central part of the aboral surface of an animal submerged in water, it is spread over that surface in radial directions and this spread is dependent upon the movement of the spines of the surface.

When an animal in process of burying itself is observed through very shallow sand from the under side, the spines are seen to exhibit a striking activity. Waves of spine movement begin along the anterior border and sweep backward over the aboral surface of the animal toward the mouth and along the lateral edges of the test to the posterior region. These waves pass in one direction only. They are the result of coördinated movements of the spines, each one of which carries out a relatively complex stroke. Apparently the individual spine swings on its facet from anterior to posterior in a vigorous vertical stroke. It returns to its initial position not by a simple reversal of this movement but by a sidewise swing in a plane near that of the general surface of the test until it has reached its starting point, when a second vertical stroke takes place followed by a lateral recovery. This can be demonstrated in part by placing an inverted sand-dollar in water shallow enough barely to cover its recumbent spines. Under such circumstances waves of spine movements can be seen passing over the anterior oral surface, and the tips of the spines as they cut the water in these waves appear always to be moving posteriorly. No spine tips in the return stroke are seen, for this is accomplished entirely under the water. These coördinated movements of the spines on the oral surface are the occasion of the animal's locomotion. Forward progress, burrowing, and righting movements of the sand-dollar are to be explained by the vigorous and well coördinated movements of these particular spines. The longest and most active spines of the anterior part of the oral side are the chief means to this end. The ambulacral feet are at best only a weak supplement to these movements.

Summary

1. The integumentary cilia in *Echinarachnius* cover the tips of the short spines and the sides of the long ones. On the oral surface they beat radially; on the aboral they beat from anterior to posterior. They play no essential part in the locomotion of the animal, but are probably concerned with feeding, with cleaning the outer surface, and with the respiratory currents.

2. The ambulacral feet form five complicated radial bands on the oral and the aboral sides of the test and a complete marginal fringe. Their tips are deep pink and provided with suckers. They are significant in locomotion to only a limited extent in that on the anterior edge of the test they pile up the sand on the aboral surface.

3. Spines cover the oral and aboral surfaces. They are of two types, long and short. They are best developed over the anterior portion of the oral surface where their distribution exhibits bilateral symmetry in relation to the axis of locomotion. In this region waves of coördinated spine movement pass from the anterior edge of the test posteriorly. In these waves each spine makes a vigorous posterior thrust in a vertical plane and an unimpeded recovery in a plane more nearly lateral. Forward locomotion, burrowing, and righting are types of motion dependent primarily on these spine movements.

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STROMBIDIUM CALKINSI, A NEW THIGMOTACTIC SPECIES

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Many ciliated Infusoria belonging to different groups can settle in a more or less temporary manner by means of their ciliated apparatus; this property is connected with some structural particularity, for example in Infusoria of the family Ancistridae and also in *Ancystropodium Maupasi*, an hypotrichous ciliate. Almost all the oligotrichous Infusoria of the family Halteriidae are planktonic species; yet some of them present occasionally thigmotactic properties and can stick or slide on the surface of solid bodies. The species of the genus *Tontonia* use, in this case, their curious caudal process lined by some small cilia; on the other hand, *Strombidium urceolare* Stein uses, according to Maupas, three long cirri located on the left side of the peristome; these cirri are fringed at their free ends whereby the Infusoria are temporarily fixed.

W. v. Buddenbrock (1922) has described under the name of *Strombidium clavellinae* a small species found in Heligoland which can either swim freely or slide on the mantle's surface of *Clavellina lepadiformis* with which this infusorian is an habitual commensal; it can also fix itself at the bottom of a dish. The fixing organ is here once more an apparatus of ciliated origin, made by four thin membranelles belonging to the left side of the adoral zone but different from the others in a considerable lengthening and in the fringed structure of their thinner ends. During the summer of 1929, I observed at Woods Hole another species of thigmotactic *Strombidium* able to fix itself temporarily by a ciliated apparatus: I will describe it under the name of *Strombidium Calkinsi*, sp. nov.

Strombidium Calkinsi is a species very nearly related to *S. sulcatum* Clap. and Lachm. and to *S. lagenula*, differing, however, from these by the presence of two long dorsal membranelles independent of the peristomal zone, the ends of which can stick on solid bodies. This infusorian measures about 40 μ in length; its body is irregularly ovoid with a great antero-posterior axis; the basal region, hemispheric, is bounded by a transversal depression; the ventral peristomal groove is lined on the right by a vertical lip extending itself on the anterior side by a so-called semicircular collar which turns dorsally toward the left.

The adoral sinistral zone takes its origin in the gullet at the bottom of the peristomal groove; this zone surrounds the collar with fourteen great membranelles. The peristomal groove, more developed than in *S. sulcatum* and less deep than in *S. lagenula*, extends toward the middle of the ventral face.

Just as in the two previous species, the posterior pole is indicated neatly not only by a transversal furrow but also by a refractive outline; this is due probably to the presence of a cuticle thicker than on the other parts of the body. A conical bundle of intracytoplasmic radiating rods fills the basal part of the body and clearly delineates the annular furrow; the significance of this formation, which exists in almost all species of *Strombidium*, is much discussed. There is no evidence in *S. Calkinsi* that the rods can act as trichocysts, but they can be compared to skeletal elements.

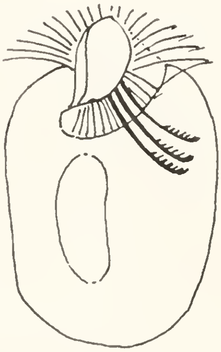

FIG. 1. *Strombidium urceolare* Stein from an original sketch of Maupas, showing "la disposition et la forme de ses longs cirres pectinés adaptés spécialement à la fixation" (Letter of Maupas, L. XI, 1907).

The ovoid nucleus lies in the body in the posterior region above the bundle of rods; the cytoplasm is hyaline, clear, enclosing various digestive vacuoles, and contains some granulations which are refractive and others which seemingly correspond to mitochondria.

The fixatory apparatus is not very easy to study when the infusorian is on the upper part of the slide. It is better to put the slide in a little dish and to look for Infusoria which are fixed on the edge and are, in this manner, seen in profile, as shown in Fig. 2.

This apparatus is constituted by two dorsal membranelles nearly as long as the body, measuring 35 to 40 μ in length and 7 to 8 μ in breadth. They appear delicately striated longitudinally and the coalescent cilia, which constitute these membranelles, separate more or less from each other at the adhesive distal extremity.

The insertion-lines of the two membranelles are rather near one another and produce two wrinkles obliquely bent from left to right, on the body's anterior dorsal side. From this point, the two membranelles spread, generally forming an angle more or less obtuse so that the points of fixation on the support can be slightly separated from each other.

The infusorian, when fixed, shows its ventral side up; the anterior dorsal membranelle is approximately perpendicular to the axis of the body and therefore to the solid support. The dorsal posterior membranelle stretches obliquely behind and often seems twisted. There is no evidence of a contractile property of the membranelles. In this fixed position the adoral zone draws around the collar half a circle perpendicular to the great axis of the infusorian's body. The vibrations of its strong membranelles produce a fluid current which flows in the ventral groove of the fixed individual. From time to

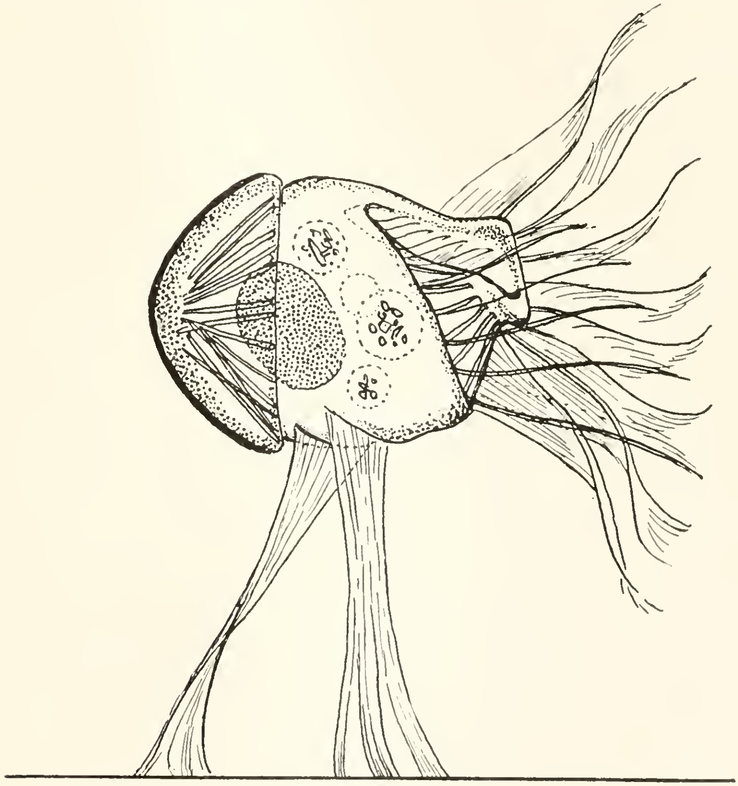

Fig. 2. *Strombidium Calkinsi* in fixed condition seen from the left side.

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time, the reaction to the current shakes the infusorian, thus showing the elasticity of the two attaching dorsal membranelles.

At times, the infusorian seems to be walking on the solid surface like an hypotrichous infusorian, the two membranelles moving one after the other, but with no regular rhythm.

More often the *Strombidium* unfastens all at once and swims hastily, drawn forward by movement of the adoral zone. In this case the two dorsal membranelles bend along the body, showing a crumpled aspect.

It is of consequence to point out that the two dorsal attaching membranelles of *Strombidium Calkinsi* are absolutely independent of the ciliary adoral apparatus. The position of their insertion lines shows that they belong to a somatic ciliature, generally absent in the oligotrichous Infusoria, but which can reappear more or less modified, either in the form of small, short, faintly mobile cilia, inserted on the longitudinal lines, or else as a more or less developed "ciliary residual field," the presence of which I have indicated in various species of Tintinnidae. Some true cultures have developed on the surface of the slides which were put in tanks filled with running sea water.

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STUDIES ON THE CHEMICAL NEEDS OF AMŒBA PROTEUS: A CULTURE METHOD

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INTRODUCTION

The chemical needs of plants have been fairly well worked out by the culture method, but knowledge regarding the needs of animals is very inadequate. This is largely due to the fact that plants can be grown in synthetic solutions containing only inorganic salts, while animals require in addition some organic material. For example, *Amœba proteus* feeds on *Chilomonas paramecium; Chilomonas* in turn requires some organic nutrient.

In the culture of protozoa, the organic nutrient has usually been added in the form of timothy hay or grain. These substances, however, contain a considerable amount of physiologically active salts, which diffuse out into the culture and alter it in an unknown way. Under these conditions, since the kind and concentration of chemical elements are not known and do not remain constant, it is difficult to ascertain the kind and number and relative amount of elements necessary for the maintenance and growth of animal protoplasm. By a method to be described below, variation in the salt composition of the medium is fairly accurately controlled, thereby making it possible to ascertain the relative importance of individual elements in rhizopod protoplasm.

METHODS AND RESULTS

Amœba proteus (Leidy) was used in all the observations made. It was derived from stock cultures made by adding a grain of rye to a mixture of half-spring-half-distilled water in finger bowls with subsequent inoculation with amœbæ and *Chilomonas.*

Two series of experiments were performed in which variation in the salt composition of the medium was controlled. These experiments were made as follows: Five balanced salt solutions[2] were prepared as

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[1] The author is indebted to Professor S. O. Mast for helpful suggestions and valuable criticisms, especially in the preparation of the manuscript, and to the Research Corporation for financial aid.

[2] Kahlbaum (analysis grade) chemicals (except Merck's blue label $Ca_3(PO_4)_2$) and water redistilled in pyrex glass were used in all solutions. According to recent investigations by Williams and Jacobs (1931), certain brands of C. P. sodium chloride contain a toxic impurity whose destructive effect overbalances any beneficial effect the sodium chloride itself may have. It should be noted that the Kahlbaum salt, which Williams and Jacobs found to be the least toxic of the five brands tested, was used in these experiments.

indicated in Table I. The first, Chalkley solution,[3] contained nine chemical elements in the form of salts, the rest contained fewer. Then 20 cc. of the solution under consideration was put into each of five 50 cc. pyrex glass beakers. Numerous amœbæ were now removed from a stock culture and put into a pyrex glass beaker containing 25 cc. redistilled water and left a few minutes, after which all that were in good condition were transferred to another pyrex glass beaker containing redistilled water. This was repeated a third time. Then 10 ± amœbæ, washed free of culture fluid, were put into each beaker containing the different salt solutions. Specimens of *Chilomonas* were now concentrated by means of a centrifuge; they were then added to a large quantity of redistilled water and again concentrated, after which 0.2 cc. of the resulting dense culture of *Chilomonas* was added to each beaker. This organism served as food for the amœbæ.

During the process of washing in redistilled water, practically all of the original culture fluid with its unknown chemical content was eliminated from both amœbæ and *Chilomonas*. No nutrient in the form of hay or grain was added to the salt solutions in the beakers. Therefore, since the composition of *Chilomonas* was the only unknown chemical factor in these solutions and since this factor was the same in all, any variation in the vital processes in *Amœba* must be due to known differences in the chemical constitution of the solutions.

TABLE I

Chemical Composition of the Solutions Tested as Culture Media for Amœba proteus

Compound	(1)	(2)	(3)	(4)	(5)
	gram	*gram*	*gram*	*gram*	*gram*
$NaCl$	0.08	0.08	0.08		
$NaHCO_3$	0.004	0.004	0.004		
KCl	0.004	0.004		0.004	0.004
$CaCl_2$	0.004	0.004	0.004	0.004	0.004
$CaH_4(PO_4)_2$	0.002	0.002	0.002	0.002	0.002
$Mg_3(PO_4)_2$	0.002		0.002	0.002	0.002
$Ca_3(PO_4)_2$					0.002
H_2O (cc.)	1000	1000	1000	1000	1000

In both series of experiments, all of the beakers were kept in diffuse light. Each was covered with a glass plate to reduce evaporation. The temperature during the course of the tests was fairly constant and was the same for all. Observations with reference to the number of amœbæ and their physiological condition were made with a binocular microscope. The condition of *Chilomonas* was also noted. These observations were made every day or every other day during the first half of the experiments; a final observation was made several days later, at which time the experiments were discontinued. The amœbæ became quite numerous in some of the solutions and the process of counting them became increasingly difficult. The latter was facilitated by marking quadrants on the bottom of the beakers with a China marking pencil and then counting the amœbæ in each section separately.

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[3] This solution is a modification by Chalkley of one used by Drew in tissue culture work; it has proven a reliable culture medium for *Amœba proteus*.

In the first experiment three salt solutions were tested as follows: (1) Chalkley solution, (2) Chalkley solution without $Mg_3(PO_4)_2$ (Table I), and a mixture of half Sieur de Mons spring water with half redistilled water.[4] The experiment continued 21 days, but at the end of a week, specimens of *Chilomonas* were not abundant in the solutions; consequently, 0.2 cc. of a fresh, washed, and concentrated culture was added to each beaker.

TABLE II

Effect of Different Salt Solutions on Growth in Amœba proteus

Solution	Days after inoculation					Average progeny per original individual
	0	4	10	17	21	
	Number of animals present					
(1) Chalkley solution	41	72	214	446	696	16.9
(2) Same less $Mg_3(PO_4)_2$	42	72	179	249	263	6.26
Mixture of half-spring-half-redistilled water	45	75	147	267	310	6.8

The hydrogen-ion concentration of all the cultures remained between pH 6.3 and 6.6 during the experiment. The results obtained regarding the number of amœbæ present are given in Table II. Each number in the first five columns of this table is the total number of amœbæ in the five beakers of the stated solution at the stated time. Each number in the last column is the average progeny produced from each original amœba by successive fissions during the course of the experiment; it is obtained by dividing the number in column five by the corresponding number in column one.

This table shows that there was growth in each of the three solutions, as indicated by increase in the number of individuals, and that

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[4] Ordinary spring water diluted with distilled water is usually a favorable medium for culturing amœbæ. In this experiment water from the Sieur de Mons spring, Mt. Desert Island, was used as the control solution. This spring water is a very weak solution of various salts ordinarily found in soil. Chalkley solution, however, was found to be a more favorable medium.

Amœba proteus can live and reproduce for more than 21 days in a synthetic salt solution with *Chilomonas paramecium* the only organic material present. It indicates that Chalkley solution is the most favorable, and Chalkley solution without $Mg_3(PO_4)_2$ is the least favorable of the three tested. The results obtained with spring water are of interest only in comparison and need not be considered further.

In the second experiment four solutions were tested in the same way as those in the preceding experiment. These salt solutions are described in Table I; *i.e.*, (1) Chalkley solution, (3) Chalkley solution without potassium salt, (4) Chalkley solution without sodium salts, and (5) Chalkley solution with the sodium salts replaced by calcium tribasic phosphate. The hydrogen-ion concentration of (1) and (3) remained between pH 6.4 and 6.6 and that of (4) and (5) between pH 6.2 and 6.4 during the 17 days of the experiment. The omission of sodium bicarbonate in (4) and (5) is responsible for this difference in hydrogen-ion concentration.

TABLE III

Effect of Different Salt Solutions on Growth in Amœba proteus

Solution	Days after inoculation					Average progeny per original individual
	0	2	4	6	17	
	Number of animals present					
(1) Chalkley solution	50	97	225	563	1956	39.1
(3) Same less potassium salt	50	85	210	565	1686	33.7
(4) Same less sodium salts	50	80	210	577	2404	48.1
(5) Same with sodium salts replaced by $Ca_3(PO_4)_2$	50	94	233	656	2676	53.5

The results obtained regarding the number of amœbæ produced are presented in Table III. The numbers have the same significance as in the preceding table. By referring to this table, it will be seen that there was little variation in the number of amœbæ present in the different solutions during the first part of the experiment but that there was considerable variation during the last part.

This variation on casual observation may not appear great, but when subjected to statistical analysis, it shows certain relations of some significance. Since the data were obtained on cultures (*i.e.*, groups of ten amœbæ) instead of on individuals, the mean progeny in five cultures of a solution was chosen as the relative value for that solution. The means with their probable errors and the difference between the means with their probable errors, computed from the same data as Table III, are given in Table IV.

It is considered reasonably certain that the mean of several measurements falls within three times its probable error; *i.e.*, the chances are 22.5 to 1 that it does. Table IV shows that the means of cultures (1) and (3) and also those of (4) and (5) are separated by about twice the sum of their probable errors whereas those of (1) and (4) are separated by more than four times the sum of their probable errors. Computation of the difference between the means shows the same relations; the difference between the means of (1) and (3) is 2.73 times the probable error, that between (4) and (5) 1.73 times, and that between (1) and (4) 5.25 times. It will be noted also that when the other solutions are compared with Chalkley solution, each of the differences between the means is probably significant, *i.e.*, in comparing (3) with (1), it is 2.73 times the probable error, (4) with (1) 5.25 times, and (5) with (1) 4.19 times. This indicates that the difference between (1) and (3) and also between (4) and (5) may or may not be significant of an actual difference between the sample distribution in these solutions but that the differences between (1) and (4) and also between (1) and (5) are significant of actual differences and cannot be due to random sampling alone. Comparison of the distribution in the different solutions by the χ^2 method (*see* Pearson, 1914) indicates also that for any two solutions the variation in the number of amœbæ present cannot be due to random sampling alone. Consequently, since the cultures were set up with the same care, received the same treatment, and differed only in the salt content of the solutions, the observed variation in fission rate must be due, at least in part, to difference in the chemical composition of the solutions.

TABLE IV

Means and difference between the means of number of amœbæ present in the different solutions. Solutions arranged in ascending order of means. Based on same data as Table III.

Solution	Mean amœbæ per culture with p.e.	Difference between means with p.e.	$\frac{M_1-M_2}{\text{p.e.}}$
(3) Chalkley less K-salt	337.2±15.55		
		54.0±19.76	2.73
(1) Chalkley	391.2±12.26		
		89.6±17.06	5.25
(4) Chalkley less Na-salts	480.8± 7.03		
		54.4±31.47	1.73
(5) Chalkley {less Na-salts plus Ca-salt	535.2±30.70	144.0±34.33	4.19

The data presented in Table III indicate therefore that Chalkley solution without sodium is more favorable than that with sodium, and that Chalkley solution with the sodium salts replaced by calcium tribasic phosphate is still more favorable. It indicates also that the absence of potassium is detrimental because the amœbæ in the solution without it had the lowest fission rate of all. The difference in hydrogen-ion concentration of (4) and (5) as compared with (1) and (3) may be a factor in the more rapid fission rate in the former solutions, although the optimum is generally considered to be at pH 6.6–6.7.

In all solutions the amœbæ remained in good condition during the experiments; at each observation practically all were attached and moving in monopodal or bipodal form. Little change was observed in *Chilomonas* during the first week, and they were still in fair condition at the close of the experiments, although less rounded and plump than at the beginning. Detailed studies on the structural and physiological changes in *Chilomonas* during starvation are in progress.

SUMMARY

1. *Amœba proteus* grows and reproduces for several weeks in a balanced salt solution containing potassium chloride, calcium chloride, calcium phosphate, magnesium tribasic phosphate, and *Chilomonas paramecium.*

2. They also grow in other solutions, but not so well; *e.g.*, in a solution containing sodium chloride and sodium bicarbonate in addition to the above salts.

3. The results obtained indicate that the presence of sodium is not only unnecessary but actually detrimental while that of magnesium and potassium is favorable if not essential for growth and reproduction in *Amœba proteus.*

4. By observing the effect on fission rate and other physiological processes of omitting various elements or altering their concentration in the solution, it is possible to ascertain the relative importance of the elements to the rhizopod protoplasm and the number and kind and amount necessary for growth.

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THE FORMATION AND STRUCTURE OF THE GLOCHIDIAL CYST[1]

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Introduction

An important phase in the development of fresh-water mussels is the obligatory period of parasitism spent upon appropriate fish hosts. While superficially encysted on such hosts the tiny larval 'glochidium' transforms into a free-living juvenile mussel, more complex in internal structure but without any corresponding increase in external size. The purpose of the present communication is to record the events incident to the formation of the glochidial cyst, to describe the structure of the cyst throughout parasitism, and to examine the morphological relations existing between parasite and host to subserve metabolic functions. A preliminary report was published some years ago (Arey, 1923). Data for the hookless group of glochidia have been drawn chiefly from an intensive study of infections of *Lampsilis luteola* on the gills of the large-mouth black bass (*Micropterus salmoides*), and of *Lampsilis anodontoides* on the long-nosed gar (*Lepisosteus osseus*). Similarly, the hooked series comprised stages of *Hemilastena ambigua* on the gills of the urodele *Necturus*, and stages of *Anodonta corpulenta* on the fins of the orange-spotted sunfish (*Lepomis humilis*).

Closely graded stages of encystment are easily procured by introducing the host into a small aquarium into which ripe glochidia have been placed. Attachment follows quickly, and samples of the gills or fins bearing glochidia can then be removed at intervals as desired. Such samples of *L. luteola*, *A. corpulenta* and *H. ambigua* were fixed promptly in Zenker's fluid. The *L. anodontoides* stages were preserved in Bouin. All the material was sectioned serially in paraffin at 6 μ and stained with hematoxylin and eosin.

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[1] Contribution No. 168. Published by permission of the United States Commissioner of Fisheries. Acknowledgment is due the staff of the Fairport Biological Station for many helpful courtesies extended during the prosecution of this inquiry.

Attachment of Glochidia

The tiny bivalved glochidium (0.3 mm. or less in size) is incapable of locomotion when liberated from the maternal gill. Chance alone brings it in contact with suitable hosts. If a fin or gill filament becomes inserted momentarily between the valves so that the chimney-like hair cells are touched, the glochidium snaps shut vigorously, pinching the intercepted tissue.[2] The more delicate, hookless group of glochidia attaches to the soft gill filaments, but the sturdy, hooked glochidia can pierce the fins as well. The process of attachment may be observed on excised gill filaments or fins placed with glochidia in a watch glass under the microscope.

Hookless Glochidia.—The sharp edges of the valves cut cleanly through the gill epithelium, affording surprisingly little evidence of hemorrhage or seeping from the incision. The location of the parasite on the gill filament governs the character of the bite. Those that attach to the blade-like edge of the filament cut through the epithelium and, usually, considerable connective-tissue stroma as well.[3] This is characteristic of most well-attached larvae, for these enclose a liberal amount of the deeper tissues. After the epithelium is passed the valves continue to close, cleaving and compressing the underlying stroma. The softer tissues are cut; the tougher gill substance, especially that containing blood vessels, is merely pinched. The walls of the blood vessels and other resistant constituents are constricted at the level of the compression, and expand like an hourglass on either side (Figs. 1 and 2).

It appears that the valve rim cuts both epithelium and soft stroma with ease, but when it encounters the tougher elements, the rim buckles inward until it lies flat against the interior of the valve proper.[4]

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[2] Arey (1921). This publication contains a full discussion of the factors involved in closure.

[3] Attachment along the edges of the filament is most favorable for easy and successful encystment. Many glochidia embed deeply in the firm gill substance, sometimes even half below the surface.

[4] The mechanisms involved in the operation of both flange and hook are described in full in a separate contribution (Arey, 1924).

This serves the very practical purpose of furnishing a broad zone of contact, while at the same time the glochidium is prevented from cutting itself entirely free (Figs. 2 to 5).

Attachment to the gill lamellae is essentially similar, but as thin-walled, vascular laminae are encountered in this instance, the chief factor is compression rather than incision. In a typical case the pinched lamellae converge inward toward the approximated valvular rims.

Hooked Glochidia.—The events during the attachment of hooked glochidia are comparable to those already described for the hookless type. Gill infections are practically identical, but fin parasites may lie wholly within the epithelium. The hooks flex much as do the flanges in the other group, but their effect is more local.[4] They pierce the host tissue like tongs, and then are inturned; the tissue is thereby held firmly, while the spines which beset the outside of the hook lock it still further.

ENCYSTMENT

Generalities.—The process of attachment is completed almost instantaneously. Both incision and compression are accomplished in less than a second. As a result, the ventral edges of the valves sink somewhat below the surface level of the host tissue (Fig. 1). There next ensues a period during which the glochidium is overgrown by the contiguous cellular tissue of the host. Successive stages of this are shown in Figs. 2 to 5. The covering-in process is rapid. In summer the black bass completes its gill cysts in about 3½ hours; yet I have observed fully formed cysts as soon as 2½ hours after attachment, and well advanced stages at one hour. Excised filaments in watch glasses may encyst glochidia even quicker than under normal conditions; two hours has been found sufficient to complete the process. The response in the gar-pike is slower than in the black bass, but encystment has been observed in three hours. Lower temperatures retard the reaction proportionately.

Glochidia which attach to gill lamellae do not form cysts as readily as those on the thicker edges of the filament. This is doubtless due to the amount of material available, as will be explained presently. On the same gill the lamellar cysts may demand twice the time taken by those along the filament's edge. Lamellae adjacent to the glochidium may unite by fusion to form the basal part of the cyst, which is then roofed over in the usual way.

At first, cysts tend to be somewhat thick, irregular and unsymmetrical (Fig. 5). Within two or three days they usually become thinner, smooth contoured and even (Fig. 6). My observations are in complete agreement with Young (1911) on this point; it is strange that Schierholz (1889), Faussek (1901) and Harms (1907) have all described the cyst as originally thin and only gradually gaining thickness. When glochidia acquire a weak attachment and clasp but a small shred of host tissue, encystment is commonly unsuccessful and the glochidium is lost.

The Method of Cyst Formation.—It is natural to assume that direct proliferation of the cells of the host tissue provides the material for the cyst that encloses the glochidium. Indeed, this assertion is presented as the correct explanation of encystment by several observers (Young, 1911; Lefevre and Curtis, 1912; and still earlier workers). That such an explanation is both inadequate and contrary to fact has been the topic of another publication by the present writer (Arey, 1932*a*). The *a priori* argument against encystment through cell multiplication rests on several facts: (1) The cyst may be composed of several thousand cells; (2) the time required for the formation of a cyst under favorable conditions is only three to four hours; (3) the mitotic cycle is relatively slow and consumes several hours in cold-blooded vertebrates.

Actual observation of encystment stages does not show the presence of more than the ordinary number of random mitoses seen in control, uninfected tissue. For example, in 78 cysts of *Lampsilis luteola* on the black bass, representing stages between 30 minutes and 9 hours after attachment, a total of only 20 positive mitotic figures and 14 doubtful ones were found as the result of a thorough census under the highest magnification. Again, in 17 cysts of *Hemilastena ambigua* on the gills of *Necturus* only one mitotic figure occurred during the period of encystment.[5] These results definitely disprove the theory of encystment through the proliferation of new cells.

Turning now to the real factor underlying encystment, the natural alternative method is actually encountered. This process is one of cell migration, whereby neighboring host cells assemble and actively push forward over the invader until the wound is closed and the glochidium is covered in.[6] After encystment is complete there may be a compensatory period of cell division in the vicinity of the cyst to replace the cells lost during the cellular emigration leading to cyst formation. For the details of this process, and its relation to wound healing in general, the reader is referred to the complete publication already mentioned (Arey, 1932*a*).

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[5] My material did not include stages beyond cysts three-fourths completed.

[6] In gill infections on fishes the cyst wall is composed both of epithelium and connective tissue (Figs. 5 to 8). Goblet cells or pigment cells are frequently carried along into the cyst. (Figs. 5 to 8).

The Structure of Cysts

The cyst is repeatedly designated as 'epithelial' by authors who have written on these matters. This, however, expresses only a half truth. Fin parasites, to be sure, may lie entirely within the stratified epithelium, and the same is true for some of the *Hemilastena* cysts on *Necturus* gills. But even in these cases there is commonly attachment to fin rays or connective-tissue stroma which necessitates a more or less extensive defect in the epithelial covering at its base.

Parasites on the gills of fishes usually bite deep into the stroma. Not only does connective tissue adjoin the glochidium here but it is carried up into the roof of the cyst as well, so that the larva in reality lies embedded in stroma (Figs. 2 to 4).[7] Often the epithelium forms a mere external arching canopy. The demarcation between epithelium and cellular connective tissue is commonly very indistinct, and the latter is easily mistaken for the former.[8] Doubtless this circumstance accounts for the existing confusion and erroneous statements concerning the composition of the cyst wall, for in some locations the interpretation is indeed puzzling and the two do appear to blend. Yet

EXPLANATION OF PLATE

Abbreviations

a.m., adductor muscle of glochidium
c.t., connective tissue of cyst
c.w., cyst wall
ep., epithelium of cyst
f., flange of valve
g.c., goblet cell
g.f., gill filament
gl., glochidium
h.t., host tissue
l.m., larval mantle

Fig. 1. Glochidium of *Lampsilis luteola* just attached to a gill filament of the black bass. Photo. × 150.

Fig. 2. An early stage in the encystment of *L. luteola* (30 minutes after attachment). Photo. × 300.

Fig. 3. A half-formed cyst enclosing *L. luteola* (1¼ hours after attachment). Photo. × 300.

Fig. 4. A cyst nearly completed about *L. luteola* (2¼ hours after attachment). Photo. × 300.

Fig. 5. The complete encystment of *L. luteola* (3½ hours after attachment). Photo. × 300.

Fig. 6. The wall of a *L. luteola* cyst at five days. Photo. × 355.

Fig. 7. The wall of a *L. luteola* cyst at four days. Photo. × 700.

Fig. 8. Tangential section of the wall of a large *L. luteola* cyst on a black bass with acquired immunity (21 hours after attachment). Photo. × 370.

Fig. 9. Gill filament of the black bass. Notches indicate the former location of sloughed *L. luteola* glochidia. Photo. × 19.

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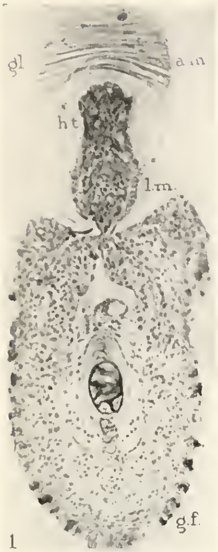
[7] Deep-lying melanophores have been found in the cyst wall. In some cysts, especially those associated with immunity, eosinophils also invade the stroma (Arey, 1932*b*).

[8] Mallory's connective tissue stain does not differentiate these tissues in fishes.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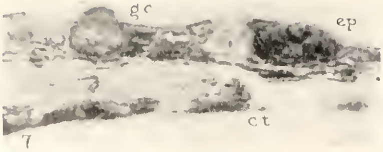
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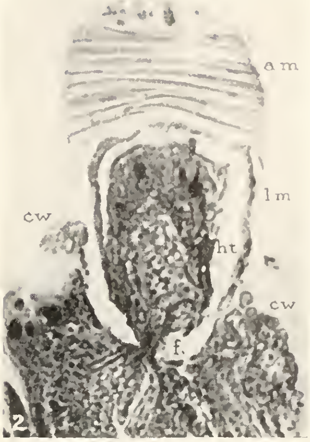
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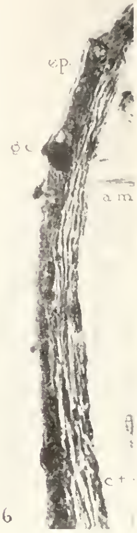
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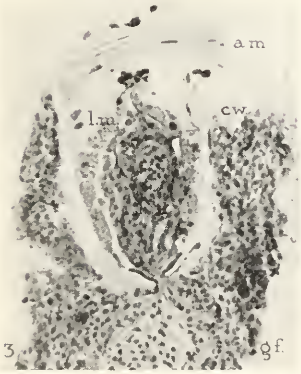
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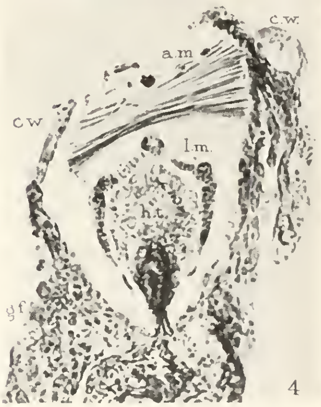
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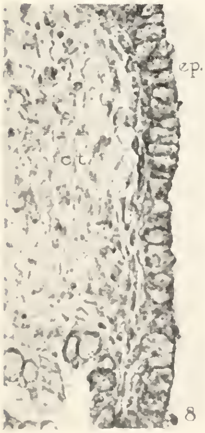
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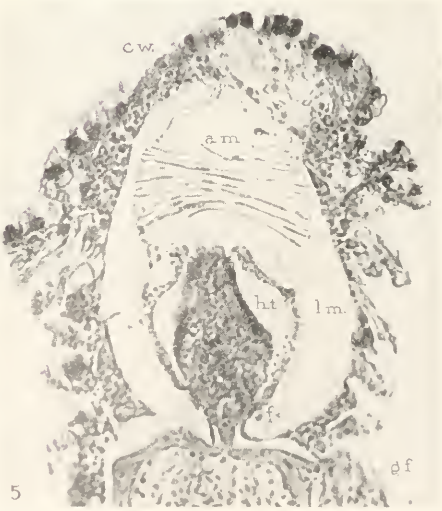
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in favorable preparations the demarcation is clear (Figs. 6 to 8); in the *Lampsilis anodontoides* series on the gar this differentiation was especially evident. Corroborative proof lies in the fact that delicate blood vessels may course through the cellular stroma, and in immune animals eosinophils wander freely through it (Arey, 1932*b*).

Correlative to these findings, the propriety of the term 'cyst' may be questioned as an exact designation for all glochidial investments. If by 'cyst' is meant a distinct envelope which demarks the larva from the adjacent tissues, then such does not exist. In the fin parasites the glochidium simply lies buried in the epithelium, or partly in the connective-tissue stroma. In the gill parasites the position is primarily in the stroma, with a roof-like canopy of epithelium outside. Yet the term is so thoroughly established and convenient as to make its replacement unwise.

The original irregularities of the cyst (Fig. 5) smooth over, and after a few days the roof tends to appear stretched and compact (Figs. 6, 7 and 9). Usually the distinction between epithelium and connective tissue then becomes plainer (Figs. 6 to 8). The goblet and pigment cells carried up in gill infections persist there (Figs. 6 and 7).

Relation of the Glochidium to its Host

Since the glochidium cannot metamorphose except on appropriate hosts it might be thought that special nutritive relations are established between parasite and host, and that this results in recognizable morphological changes or adaptations in the enveloping tissue. As a matter of fact, this possibility is not realized (Figs. 6 to 8). The soft, and for the most part highly vascular, tissues in which the parasite is embedded are apparently adequate for handling whatever interchanges are necessary without any special elaborations. The host tissue ingested at the time of attachment, together with the degenerating larval adductor muscle, are important sources of nutriment during transformation (Arey, 1932*c*), so that there is no metabolic 'strain.' The adjoining host tissues do not become unusually vascularized (Figs. 7 and 8) except in the *Proptera* glochidial type which is peculiar in that it undergoes marked increase in size during a postmetamorphic period of retention. In some specimens of *Proptera laevissima* which had increased in bulk some 40 times, the cysts were found to be very large and thick, and capillaries were present that presumably represented secondary invasive growths.

The Rupture and Repair of Cysts

When the cyst first forms, its wall is regionally variable in thickness and usually bears irregular outgrowths (Fig. 5). After a day or two it becomes smooth and quite symmetrical. The tissue over the top gradually assumes a compact and stretched appearance, and the wall as a whole is thinner (Fig. 6). This reduction and thinning is much more spectacular in the bulky cysts which characterize the brief attachment of glochidia on immune hosts or non-hosts. In a contribution (Arey, 1932*b*) specifically describing these conditions it will be shown that the thinning is apparently due to the removal of cells back into the filament, rather than to a loss by desquamation or otherwise.

After the first days of encystment there are no especially significant changes in the cyst until the time when the glochidia are shed. Young (1911) has described a characteristic loosening of the cyst tissue and a concomitant infiltration of lymph after about one week of parasitism. A mild degree of cellular separation, in which intercellular bridges become prominent, occurs also in some of my series. Nevertheless, this is by no means a regular phenomenon, while sometimes it is observed relatively distant from an encysted glochidium as well. To what extent such alterations are artefacts and what their proper interpretation may be must remain unanswered at present. Miss Young suggested a causal relation to the premature sloughing of partly transformed glochidia. This may be true, but if so it is not a characteristic method by which these parasites terminate a normal period of encystment.

At the end of the parasitic period the glochidium becomes free of the host.[9] It has not increased in external size, but internally the metamorphosis is marked. Liberation is partly the result of the young mussel's own activity, for at intervals prior to detachment the valves may be observed to move and the foot to be pushed about, pressing the cyst wall. This is demonstrable when at this time filaments are removed and kept in watch glasses under a microscope; incidentally, there is reason to suspect that emergence is accelerated by such *in vitro* procedure. The cyst is eventually ruptured, but sections do not show that this is made perceptibly easier by any sudden terminal thinning or weakening of the wall. Portions of the old cyst-covering may be carried away and adhere for a time to the freed glochidium. Apparently a certain amount of gross sloughing aids the shedding process, for when infections are made on immune fish, transformation of the glochidium fails and the passive glochidium is liberated while still encysted (Arey, 1932*b*).

The freeing of the transformed glochidium leaves a defect in the filament which is rapidly filled in (Fig. 9), probably by the same sort of cell mobilization that characterized encystment. Sections are not particularly informative on this point, and even the sites of the cysts are not easily detectable in microscopic preparations. In some instances the cavity of the remnant of the former cyst is temporarily filled with a coagulable exudate.

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[9] Those at the tips of gill filaments are often retained longest (Fig. 9). This is conceivably due to their less favorable position for receiving nutrition or oxygen.

SUMMARY

Mechanical stimulation of the larval glochidium induces nonselective, automatic closure upon the impinging gill or fin. The valves largely cleave the soft tissues encountered, but merely clasp such tough elements as blood vessels and fin rays which lie deeper. As a result, part of the glochidium is buried in host substance.

The glochidium is then covered by host tissue which advances from all sides, primarily for the purpose of closing the wound. Encystment is not the result of cell proliferation. On the contrary, it is accomplished by a mass movement of cells from the adjoining regions, advancing by their own activities and directed over the exposed valves by thigmotaxis. A compensatory period of mitosis may appear subsequent to encystment, apparently to replace cells lost to the cyst by emigration.

Fin cysts are largely epithelial in structure. Glochidia which attach to gill filaments lie embedded in cellular connective tissue, roofed over with an epithelial canopy.

Shortly after encystment is completed the cyst becomes thinner, smoother and more symmetrical. Thereafter, and even until the time of rupture, there are no further significant morphological changes in the cyst. Special adaptations of the host tissues to care for the wants of the metamorphosing parasite are not developed.

The glochidium is liberated partly through its own efforts, apparently aided somewhat by sloughing. Repair of the resulting defect in the host tissue is rapid and probably follows the general method utilized at encystment. This would involve an early redistribution of existing cellular elements, followed later by the formation of new cells to restore the tissue balance.

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THE

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A STATISTICAL TEST OF THE SPECIES CONCEPT IN LITTORINA

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> "One may believe that if larger series were more often utilised in taxonomic work the current bewilderment over variation would give way to a renewed respect for a certain uniformity that exists thruout such groups of individuals."—Kinsey.

With certain exceptions, no two individuals of a species are ever genetically identical; hence it is not so much the uniformity as the character and range of variation in a species that are diagnostic. Conversely, the fact that two related animals differ does not necessarily mean that they belong to separate species unless it can be shown, after the examination of sufficient numbers collected over a wide area, that there is not a series of overlapping intergrades between the two differing forms.

It was, for example, the range in variation in the number of vertebræ in conger eels that enabled Johannes Schmidt (1931) to separate the American species, *Conger oceanicus*, from the European *C. vulgaris*. The ranges of the larvæ of the two species overlap in part geographically, but not anatomically, the number of vertebræ in *C. oceanicus* being from 140 to 149, average 144.63, and in *C. vulgaris* from 154 to 163, average 158.16. This lack of overlap in the numbers of their vertebræ clearly justifies their segregation.

Kinsey (1930) has shown, too, that the highly variable Gall Wasp, *Cynips erinaceus*, is one species, though extreme forms of its gall have been previously assigned to separate species. In any part of its wide range a comprehensive collection over a square mile will very closely resemble a similar comprehensive collection at any other place in the insect's range. The variation in the species is roughly constant throughout its entire geographic range.

These are two extreme cases; the first of two distinct, the second of one homogeneous, species. If, however, the two conger eels had numbers of vertebræ ranging from 140 to 152 and from 150 to 163 respectively, it would be impossible from the vertebræ alone to assign to either group those possessing 150, 151 or 152 vertebræ. To get round this difficulty, one man would consider them mere races, another sub-species, and a third separate species with a certain amount of hybridization, the choice depending on the taxonomic upbringing and prejudices of the worker.

Again, if in such a case as *Cynips erinaceus* the amount of variation were not constant, a comprehensive collection from one part of its range would be different from a similar collection from another part. If the variations in these two collections did not overlap, they might be considered separate species unless they were merely the ends of a continuous series.

This last is the case with *Littorina obtusata*. There has been but little uniformity in the treatment of its varying forms, and the resulting taxonomic muddle must be first of all cleared up.

The establishment of the genus *Littorina* has always been ascribed to the elder Férussac, the reference in the Index Animalium (Sherborn, 1927) being to page xxxiv of the Tableaux systématiques généraux de l'embranchement des Mollusques (Férussac, 1822). This refers, however, only to where *Littorina* is listed as the fifth sub-genus of the genus "PALUDINE, Paludina, FÉRUSS. (Fluv. et marin.)," with no definition or description. The definition, such as it is, is given on page xi, where Férussac says: "Quant aux paludines marines qui constituent le genre Trochus d'Adanson, comme nous n'avons pu adopter cette dénomination à cause des *trochus* de Linné (en general formés d'espèces réellement congénères, ce qui nous a empêché de les appeler *turbo* avec Adanson, en y rapportant tous les *turbo* de Linné qui doivent s'en rapprocher), nous en formons un sous-genre sous le nom de *littorine*." (No Latin is used here, but an objection on that score would, I think, be oversteeped in pedantry, since only the final *e* needs altering.) Two pages before, at the bottom of page ix, Férussac gives a list "dressé d'aprés l'édition de Gmelin," referring to the thirteenth edition of the Systema Naturæ, edited by J. F. Gmelin (Linnæus, 1788). This list, compiled from the Linnæan genera *Trochus* and *Turbo*, which Férussac wished to combine, includes five species referred to as "*Paludina* Marine." These are *obtusatus*, *neritoides*, *littoreus*, *muricatus*, and *afer*, constituting his sub-genus *Littorina*.

"Le genre Trochus d'Adanson," mentioned above, consists of four species described and illustrated in the Histoire Naturelle du Sénégal (Adanson, 1757). Their names, on a binomial system of Adanson's own devising, antedate the tenth edition of the Systema Naturæ (Linnæus, 1758) by one year, and therefore do not stand. These species are beautifully illustrated in Adanson's work, and undoubtedly belong to the genus *Littorina*.

Menke, 1828, was responsible for the spellings *Litorina* and *litorea*, although both Linnæus and Férussac used two *t*'s. Menke said that the alteration made for correct Latin, which is true, but it also led to a small confusion in nomenclature which has lasted till this day. In truth, "optima nomina quæ nihil significant."

The first full definition or description of the genus *Littorina* is given in the second edition of Lamarck's Histoire Naturelle des Animaux sans Vertèbres, Vol. XI, pp. 201 et seq. (Lamarck, 1843).

Of the British Littorinas, *L. littorea* and *L. neritoides* are both described under the genus *Turbo* in the tenth (1758) edition of Linnæus' Systema Naturæ, and they have retained their original specific names and authority, except in the work of a few authors who have fortunately caused no lasting confusion, save for the fashion, introduced by Menke (1828), of spelling with one *t*.

The nomenclature of the mollusks generally known by naturalists as *L. obtusata* and *L. rudis*, however, has for a long time been a field for error and dispute.

Littorina rudis is assigned by the Marine Biological Association (1931) to Maton, quoting Jeffreys (1865). Johnson (1915) gives *L. rudis* (Donovan), quoting Gould (1870) and Donovan (1804) (whose date is erroneously given in Gould as 1800, the date of publication of the second volume; the first volume was actually published last, in 1804, after the fifth in 1803). Kuester (1856) calls the unfortunate animal *L. rudis* (Montagu), quoting Montagu (1803) and Maton and Rackett (1807), in spite of the fact that the latter authorities cite Maton (1797) as the originator of the name and description; Jeffreys (1865) also gives Maton. Forbes and Hanley (1853), however, mention Donovan (1804), though Maton and Rackett (1807) give Maton (1797), Montagu (1803) and Donovan (1804) in order of priority. Montagu disturbs this order by quoting from the then unpublished volume of Donovan, having presumably seen the manuscript or proofs, but Donovan nevertheless gives the credit of the name *rudis* to Maton (1797). Menke (1830), the first to put *Turbo rudis* into the genus *Littorina*, cites Montagu (1803) as author of the species.

Under the Law of Priority, the name *rudis* must be referred to Maton (1797), since there is no doubt of the identity of the form he described.

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However, *rudis* is not the correct specific cognomen of the snail which usually passes under that name. In 1792 Olivi published a description and rough figures of a shell near Venice which he called *Turbo saxatilis.* Jeffreys (1865) states that this form is identical with *Littorina neritoides* (L.), but Dautzenburg and Fischer (1912) present on two plates sixty-two exquisitely colored and enlarged figures of *L. saxatilis* and *L. rudis*, which show that *L. rudis* is specifically indistinguishable from *L. saxatilis*, of which names the latter has the priority by five years (1792 and 1797).[1]

In the case of the snail variously known as *Littorina obtusata*, *L. littoralis* and *L. palliata*, the difficulties begin with the tenth edition of the Systema, where two very similar shells are described under different genera, to wit, *Turbo obtusatus* (Vol. I, p. 761) and *Nerita littoralis* (Vol. I, p. 777). The early British conchologists Montagu (1803), Donovan (1804) and Maton and Rackett (1807) all accepted *Nerita littoralis* as representing the common form of the English Channel and British Coasts. Here Montagu introduces a minor confusion by claiming that *Nerita littoralis* L. is the same as *Turbo neritoides* L. He is alone in this opinion, and it is difficult to see what led him to form it. In 1822 Thomas Say described *Turbo palliatus* from the coast of Maine, and this is considered by both Dautzenburg and Fischer (1915) and Johnson (1915) to be a variety or sub-species of the *Turbo obtusatus* of Linnæus.

Jeffreys (1865) gave the specific name *obtusata* to the form on the shores of the English Channel, evidently assuming the identity of the two Linnæan shells. Dautzenburg and Fischer (1915) regard the English Channel (*littoralis*), the Norwegian (*obtusata*) and the New England (*palliata*) forms as varieties or subspecies of the one species, of which the prior name, by pagination in the Systema Naturæ, must be *Littorina obtusata*. Winckworth (1922), however, assigning the British Littorinas to four genera,[2] *Littorina* Férussac, *Littorivaga* Dall, *Melarhaphe* Menke and *Neritoides* Brown, includes in the last-named the three species *obtusata* (L.), *æstuarii* (Jeffreys) and *littoralis* (L.), the existence of *obtusata* on the shores of Great Britain being thought doubtful, all of which leads us to the impasse of a difference of opinion among experts.

---

[1] *L. saxatilis* (Olivi) is not to be confused with *L. saxatilis* (Johnston), which is a so-called variety from Berwick, England (Jeffreys, 1865).

[2] These generic names are regarded in this paper as only of subgeneric rank. They are extremely valuable in the finer delimitation of the genus *Littorina* as a whole, but considered as genera they only bewilder the poor naturalist and field worker.

Very full lists of synomymies are given for the *obtusata-littoralis-palliata* group by Dautzenburg and Fischer (1915) and for *saxatilis-rudis* by Dautzenburg and Fischer (1912).

This confusion is due solely to the fact that inferences about varieties have been drawn from individual specimens, and the only way to unravel this tangle is to examine a large enough number of animals until the answer to the problem ceases to be a matter of opinion.

Specimens of *L. obtusata* were therefore obtained from eleven different localities, on both sides of the Atlantic Ocean. The minimum number aimed at was one hundred from each locality, but sufficient specimens were not always forthcoming. In only one case, however, was the number less than sixty. Table I gives the localities of the lots, the number of individuals, and the catalogue numbers in either the Museum of Comparative Zoölogy, Harvard University, or the Museum of the Boston Society of Natural History.

Table I

Localities, number of individuals and catalogue numbers of eleven lots of L. obtusata.
M.C.Z. = Museum of Comparative Zoology, Harvard University.
B.S.N.H. = Museum of Boston Society of Natural History.

	Locality	No.	M.C.Z.	B.S.N.H.
a..	Bergen, Norway	100	47508	
b..	Cattewater, Plymouth, England	100	76980	
c..	Church Reef, near Plymouth, England	100	76978	
d..	Near Westerly, Rhode Island	100	13980	
e..	South Cohasset, Massachusetts	100		26655
f..	Briar Neck, Gloucester, Massachusetts	86		24787
g..	Rye Beach, New Hampshire	75		26806
h..	Broad Cove, Georges River, Cushing, Maine	38	67775	
i..	Port Clyde, Knox County, Maine	74	67773	
j..	Tenants Harbour, Knox County, Maine	60	67774	
k..	Isle au Haut, Maine	100	13972	
	Total	933		

The positions of the localities are shown in the maps, Figs. 1 and 2.

The lot *a* in Table I were kindly sent to me by Professor Brinkmann of the Bergens Museum, lots *b* and *c* by the Marine Biological Association at Plymouth, England, lots *i* and *j* by Mr. N. W. Lermond, and lots *e*, *f* and *g* were lent by Mr. C. W. Johnson of the Boston Society of Natural History. The rest, *d*, *h*, and *k*, were already in the collection of Harvard University.

Each shell was measured with callipers to the nearest tenth of a millimeter along three dimensions; the length (*a*) of the final whorl

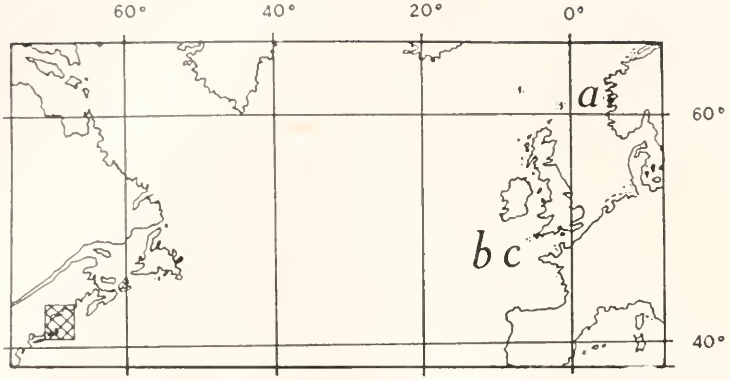
60°
40°
20°
0°
60°
40°
a
b
c

FIG. 1. Map showing the localities on the two sides of the North Atlantic. The shaded area is that shown on a larger scale in Fig. 2. The letters refer to Table I.

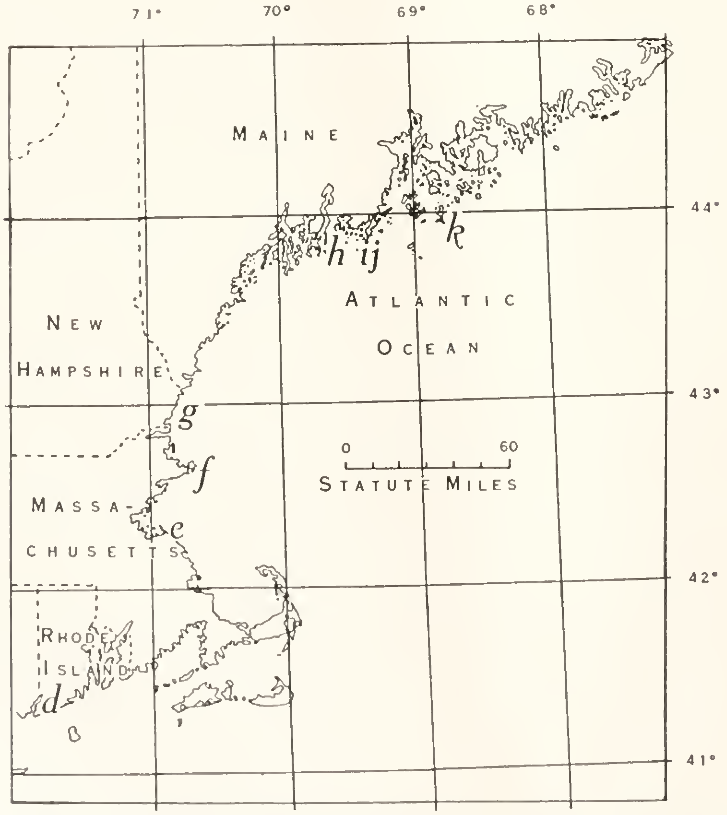
71°
70°
69°
68°
44°
43°
42°
41°
MAINE
NEW HAMPSHIRE
MASSACHUSETTS
RHODE ISLAND
ATLANTIC OCEAN
0
60
STATUTE MILES
d
e
f
g
h
i
j
k

FIG. 2. Map showing the localities on the New England Coast. The letters refer to Table I.

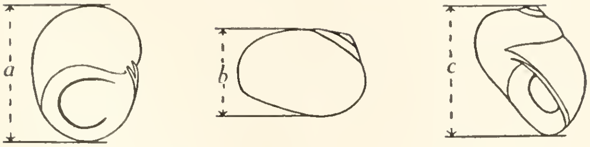

FIG. 3. Shell from Church Reef, near Plymouth, England, showing the dimensions that were measured.

(usually the overall length of the shell), the breadth (b) of the final whorl at the base of the penultimate whorl, and the distance (c) from the top of the spire to the most distant point on the lip. The ratios between these dimensions, $\frac{a}{b}$, $\frac{a}{c}$ and $\frac{c}{b}$, were calculated, and they form the basis of comparison between shells from different localities. Figure 3 shows exactly which dimensions were measured on each shell.

TABLE II

Shells from the two sides of the Atlantic: numbers of shells of different sizes. (See Fig. 4.)

Localities	Bergen			Cattewater			Church Reef			Westerly		
Number of Shells	100			100			100			100		
Millimeters	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
5.75 to 6.25.....					1							
6.25 to 6.75.....					9							
6.75 to 7.25.....					28						1	
7.25 to 7.75.....					34	1					16	
7.75 to 8.25.....					13	7					43	
8.25 to 8.75.....		2			13	21					29	
8.75 to 9.25.....		25		7	2	30		5			10	
9.25 to 9.75.....		30		17		21		30			1	1
9.75 to 10.25.....		39		21	1	12		31				6
10.25 to 10.75.....		4	1	26		4		29				33
10.75 to 11.25.....			5	14		2		5		7		27
11.25 to 11.75.....			11	9		2			1	29		18
11.75 to 12.25.....	2		21	4					1	28		9
12.25 to 12.75.....	8		35	2					8	19		5
12.75 to 13.25.....	25		19				1		15	9		1
13.25 to 13.75.....	37		7				2		17	8		
13.75 to 14.25.....	19						17		33			
14.25 to 14.75.....	8		1				22		14			
14.75 to 15.25.....	1						28		9			
15.25 to 15.75.....							20		1			
15.75 to 16.25.....							7					
16.25 to 16.75.....							2					
16.75 to 17.25.....							1		1			

TABLE III

Shells from the two sides of the Atlantic: numbers of shells of different proportions.
(See Fig. 5.)

Localities	Bergen			Cattewater			Church Reef			Westerly		
Number of Shells	100			100			100			100		
Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.99 to 1.03.......		4			1			3			1	
1.03 to 1.07.......		20			4			35			15	
1.07 to 1.11.......		52			17	1		47			53	
1.11 to 1.15.......		20			51	3		12			27	
1.15 to 1.19.......		4	1		21	18		3			4	
1.19 to 1.23.......			7		5	45			1			
1.23 to 1.27.......			29	1		22			2			5
1.27 to 1.31.......	1		33		1	7			5			12
1.31 to 1.35.......	7		18	15		1			21			32
1.35 to 1.39.......	38		10	45		2	3		22	1		28
1.39 to 1.43.......	27			26			6		34	13		17
1.43 to 1.47.......	21		2	12		1	26		7	24		5
1.47 to 1.51.......	5			1			31		7	36		
1.51 to 1.55.......							23		1	17		1
1.55 to 1.59.......	1						7			7		
1.59 to 1.63.......							4			1		
1.63 to 1.67.......										1		

It represents a shell from Church Reef, Wembury Bay, and the measurements in millimeters and the proportions calculated therefrom are as follows:

Length (*a*)	Breadth (*b*)	Spire to Lip (*c*)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
16.4	10.8	15.2	1.518	1.079	1.407

SHELLS FROM THE TWO SIDES OF THE ATLANTIC OCEAN

When shells from Bergen, the Cattewater, Church Reef, and Westerly are compared, they are found to be so similar that it is impossible to separate them.

In average size of individuals the four lots vary considerably, those from the Cattewater being the smallest and those from Church Reef, only six miles away, the largest. Among the Cattewater shells, however, there were nine very large individuals illustrated by No. 6 in Fig. 6. These presumably represent a previous generation or age-group, and they have not been included in the Cattewater shells in Tables II and III and in Figs. 4 and 5. Table III and Fig. 5, giving the numbers of shells at the different proportions, show the amount of overlap between the four lots. Bergen and Cattewater shells are very similar, and the curves for Church Reef and Westerly are well-nigh identical. The Cattewater lot are somewhat more globular than the others, but they are probably younger, if the giant shells like No. 6 are really of the mature adult size. These nine large Cattewater shells have apparently lived an exceptionally long time, for they are the largest *Littorina obtusata* in the extensive collection at Harvard, with the exception of an individual specimen from Cornwall.

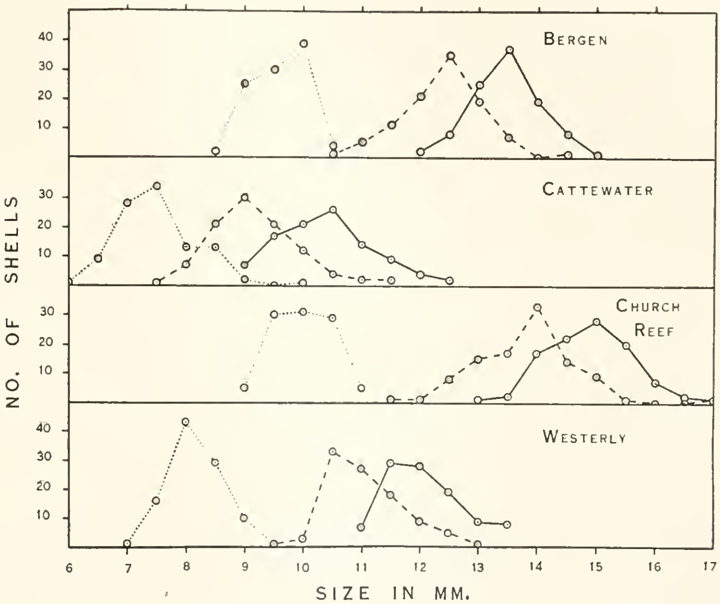

FIG. 4. Shells from the two sides of the Atlantic: numbers of shells of different sizes (See Table II). — = *a*, · · · = *b*, --- = *c*.

In Fig. 6 are shown camera lucida drawings of the obverse and reverse aspects of three shells from each locality, the relative height of the spire to the rest of the shell increasing from left to right. The dimensions and proportions of the same twelve shells are given in Table IV, where the ratio $\frac{a}{c}$ decreases in each group with the increase of the spire component, *c*.

From these figures and tables it will be seen that the amount of variation is much the same in each locality and that it would be quite impossible to sort a mixture of shells from the four places, either on sight or after measurement. There is then no doubt of the conspecificity of *Littorina obtusata* from Bergen, from the Plymouth district and from Rhode Island.

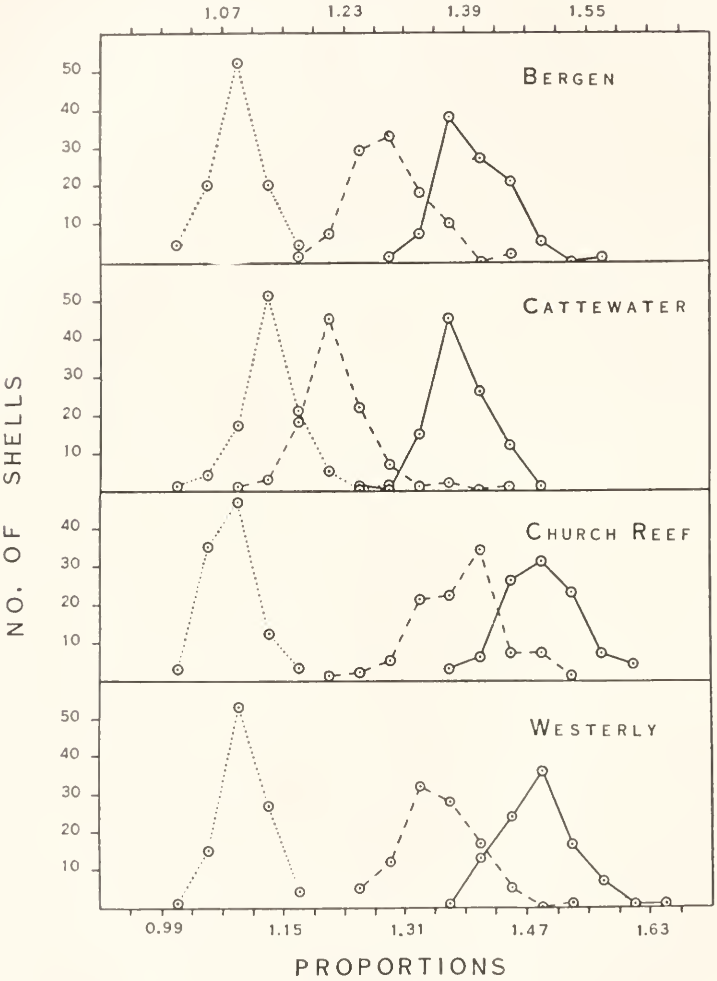

FIG. 5. Shells from the two sides of the Atlantic: numbers of shells of different proportions (See Table III). — $= \frac{a}{b}$, ··· $= \frac{a}{c}$, --- $= \frac{c}{b}$.

SHELLS FROM THE NEW ENGLAND COAST

It has now been shown that the same species occurs on both sides of the North Atlantic. It remains to be proved that the same species extends up the American Coast, where shells called "*L. palliata*" or

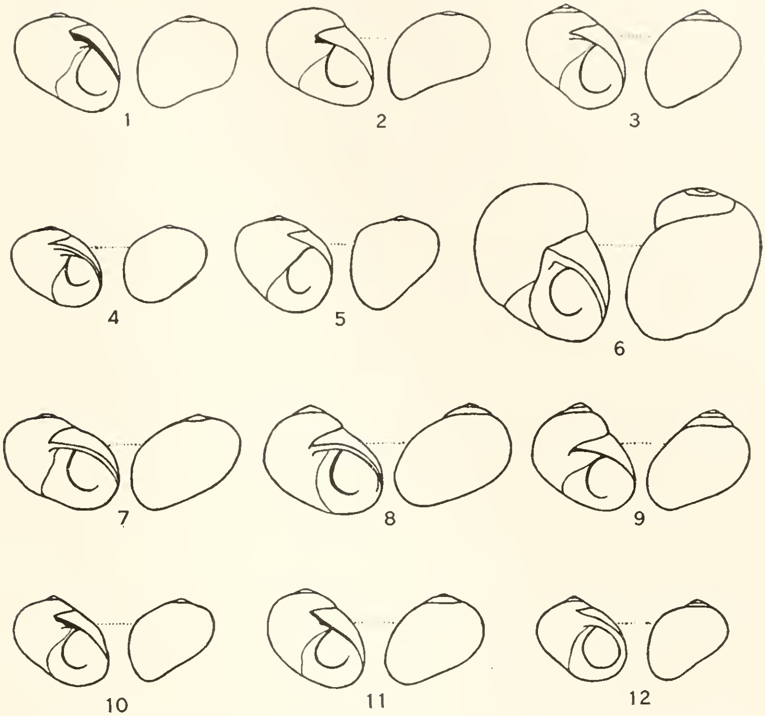

Fig. 6. Shells from the two sides of the Atlantic: obverse and reverse aspects of shells whose dimensions are given in Table IV.

Table IV

Shells from the two sides of the Atlantic: dimensions and proportions of shells illustrated in Fig. 6.

Locality	Length (a)	Breadth (b)	Spire to Lip (c)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
	mm.	*mm.*	*mm.*			
1. Bergen........	14.1	10.5	12.8	1.343	1.102	1.219
2. Bergen........	14.1	9.9	13.0	1.424	1.085	1.313
3. Bergen........	13.8	9.7	13.0	1.422	1.062	1.340
4. Cattewater.....	11.5	8.3	9.9	1.386	1.161	1.192
5. Cattewater.....	12.5	9.3	11.7	1.344	1.069	1.258
6. Cattewater.....	19.1	13.0	18.9	1.469	1.010	1.454
7. Church Reef....	14.9	9.5	12.9	1.569	1.155	1.358
8. Church Reef....	15.5	10.5	14.6	1.476	1.061	1.391
9. Church Reef....	13.7	9.1	13.5	1.505	1.015	1.484
10. Westerly.......	12.4	8.3	11.3	1.494	1.097	1.361
11. Westerly.......	13.2	9.0	12.3	1.467	1.074	1.367
12. Westerly.......	11.2	7.9	10.5	1.418	1.067	1.329

"*L. obtusata palliata*" occur from Long Island Sound to Newfoundland.

Between shells from Westerly, R. I., and Isle au Haut, Me., there is a considerable difference, namely that between the varieties *littoralis* and *palliata* as described by Dautzenburg and Fischer (1915). These are illustrated in Fig. 7, which represents shells 10 and 33 in Table VII and Fig. 10. Without intermediate forms these might well be considered separate species, the most obvious differences being the thinner shells and the taller spires of the Maine forms. If shells from intermediate places are interpolated, however, a gradual series is obtained between the two extremes, which makes any subdivision impossible. This is clearly shown in Fig. 9, in which the modes of the curves gradually pass from the arrangement in the Westerly lot to that from Isle au Haut, with no sudden transition anywhere. The numbers from which Figs. 8 and 9 are compiled are given in Tables V and VI respectively. In Fig. 10 are shown the obverse and reverse aspects of 24 shells, three from each New England locality, arranged as in Fig. 6 in ascending height of spire from left to right. The corresponding dimensions and proportions are given in Table VII.

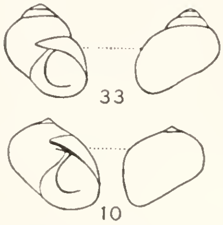

FIG. 7. Shells 10 and 33 from Fig. 10, to show the approximate limits of variation in New England shells.

Shells 10 and 11 correspond to *L. obtusata littoralis* (D. and F.), Shells 17, 18, 20 and 21 to *L. obtusata typica* (D. and F.), and Shells 29, 30, 32 and 33 to *L. obtusata palliata* (D. and F.). By Winckworth's (1922) reckoning, Shells 10 and 11 belong to a separate species, *L. littoralis*. These figures show, however, that these shells are all part of a continuous series, and any distinction into species or varieties must be purely arbitrary and taxonomically invalid.

Indeed, such bestowal of specific and varietal names and ranks on forms whose differences have not been analyzed would, if carried to the logical conclusion, necessitate the granting of a separate name to every individual (since no two are alike), with an accompanying increase in nomenclatural complexity.

TABLE V

New England shells: numbers of shells of different sizes. (See Fig. 8.)

Locality	Westerly Rhode Island			Cohasset Mass.			Briar Neck Mass.			Rye Beach N. H.			Broad Cove Maine			Port Clyde Maine			Tenants Hbr. Maine			Isle au Haut Maine		
No. of Shells	100			100			86			75			38			74			60			100		
Millimeters	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
5.25 to 5.75.....					3			1												10			4	
5.75 to 6.25.....					14			1			1									26			32	
6.25 to 6.75.....					38			4			1			2			2			18			50	
6.75 to 7.25.....		1			30			9			5			1			4			5			12	
7.25 to 7.75.....		16			13			16		1	12	1		11			8			1	1		2	
7.75 to 8.25.....		43			2	5		25	2		31			18			20		4		8			
8.25 to 8.75.....		29		8		16	1	20	3		21	2		3			27		9		12			2
8.75 to 9.25.....		10		16		28	2	7	5	1	4	2		3	2		13	5	14		14	6		18
9.25 to 9.75.....		1	1	19		21	4	2	5	2			2			4		1	16		14	30		18
9.75 to 10.25.....			6	25		19	6	1	12	1		5			5	2		3	11		3	37		38
10.25 to 10.75.....			33	20		9	10		16	3		12	11		10	1		5	3		6	20		18
10.75 to 11.25.....	7		27	8		2	7		18	17		21	4		9	6		9	1			6		4
11.25 to 11.75.....	29		18	4			21		5	21		23	16		7	10		14	2		2	1		2
11.75 to 12.25.....	28		9				13		12	15		8	2		3	17		14						
12.25 to 12.75.....	19		5				9		5	9		1				16		11						
12.75 to 13.25.....	9		1				8		2	4			1		1	13		9						
13.25 to 13.75.....	8						3		1	1			2		1	5		2						
13.75 to 14.25.....							3		1									1						

TABLE VI

New England shells: numbers of shells of different proportions. (See Fig. 9.)

Locality	Westerly Rhode Island			Cohasset Mass.			Briar Neck Mass			Rye Beach New Hampshire			Broad Cove Maine			Port Clyde Maine			Tenants Hbr. Maine			Isle au Haut Maine		
Number of Shells	100			100			86			75			38			74			60			100		
Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.87 to 0.91.......																							1	
0.91 to 0.95.......											1									1			5	
0.95 to 0.99.......					5									3			11			10			15	
0.99 to 1.03.......		1			17			5			20			16			19			18			44	
1.03 to 1.07.......		15			44			32			34			15			29			25			31	
1.07 to 1.11.......		53			33			44			18			4			15			6			2	
1.11 to 1.15.......		27			1			6			2												2	
1.15 to 1.19.......		4																1						
1.19 to 1.23.......																								
1.23 to 1.27.......			5			2			2							1								
1.27 to 1.31.......			12			9			10	1		8			2			2						
1.31 to 1.35.......			32	1		17			28			19	1		9			8			1			
1.35 to 1.39.......	1		28	8		21	5		28	5		24	3		12	4		15			3			2
1.39 to 1.43.......	13		17	24		27	21		12	24		13	17		7	7		17	2		7	2		7
1.43 to 1.47.......	24		5	24		11	27		6	32		7	11		5	24		14	5		18	3		11
1.47 to 1.51.......	36			24		8	29		1	8		3	6		2	28		10	21		14	19		17
1.51 to 1.55.......	17		1	8		3	4			4		1			1	8		5	20		9	20		12
1.55 to 1.59.......	7			8		1	1			1						1		1	10		3	20		21
1.59 to 1.63.......	1			3		1										1		1			2	17		12
1.63 to 1.67.......	1																		2		1	14		10
1.67 to 1.71.......																					1	4		4
1.71 to 1.75.......																					1	1		3
1.75 to 1.79.......																								1

As a final demonstration of the essential unity of *Littorina obtusata* over this considerable geographic range, embracing, as it does, both southern Norway and New England, the data of all the 933 shells from the eleven localities may be added together. This is done in Table VIII, and the result shown in Fig. 11. Although the chief distinction between so-called varieties of *L. obtusata* is usually the variation in the relative height of the spire, the curve $\frac{a}{c}$ in Fig. 11,

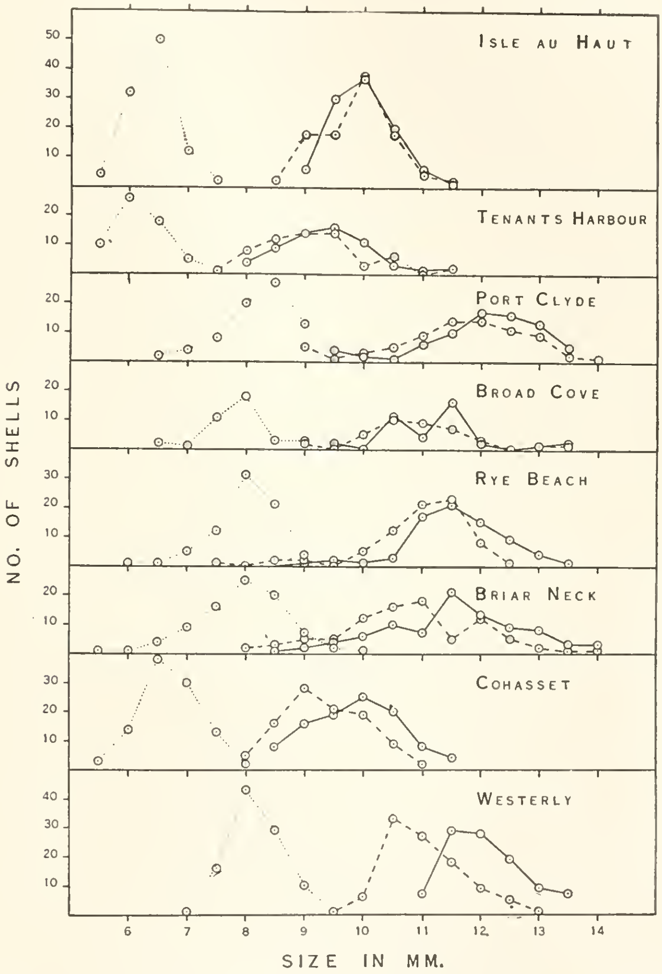

FIG. 8. New England shells: numbers of shells of different sizes (See Table V). — = *a*, ··· = *b*, --- = *c*.

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giving the ratio of Length to Distance from Spire to Lip, is an almost perfect frequency curve, because the series from blunt to elevated

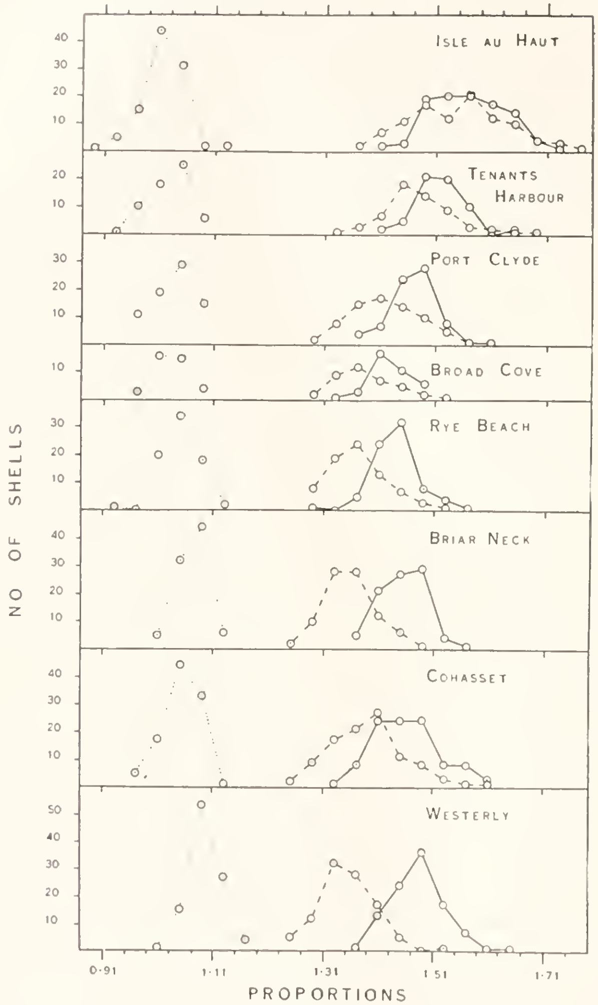
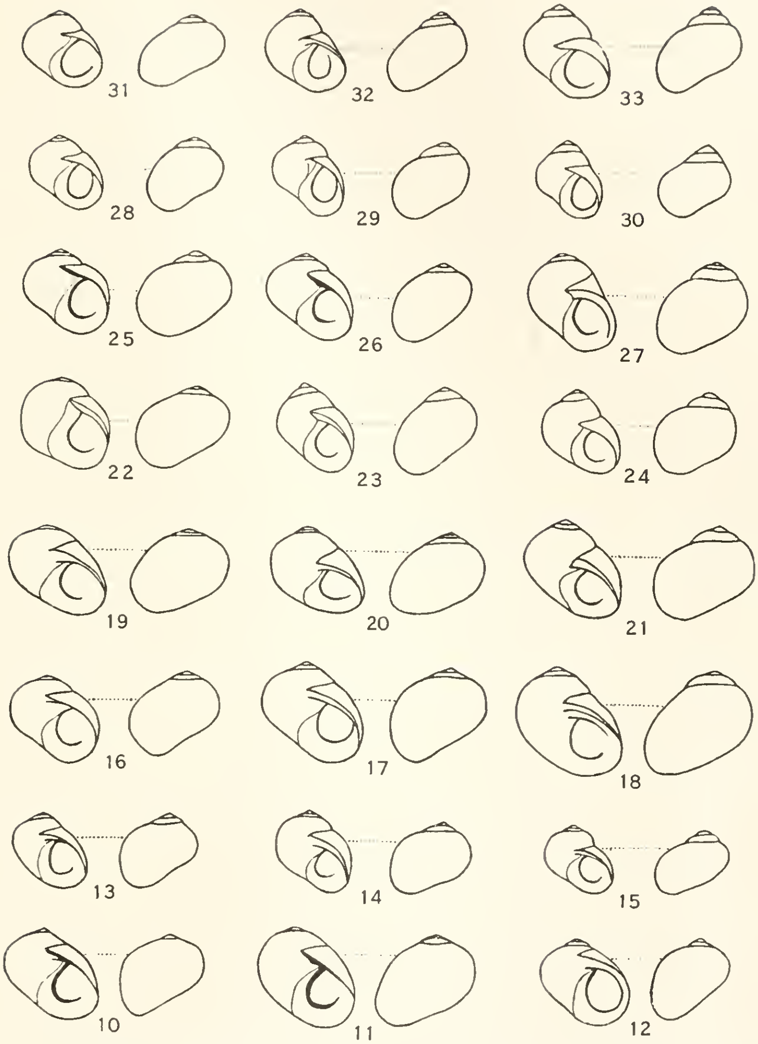


FIG. 9. New England shells: numbers of shells of different proportions (See Table VI). — $= \frac{a}{b}$, · · · $= \frac{a}{c}$, - - - $= \frac{c}{b}$.

spires is continuous, with no sudden jumps. The "average" *L. obtusata*, according to these figures, will have the proportions:

$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
1.47	1.07	1.37


31
32
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22
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21
16
17
18
13
14
15
10
11
12

FIG. 10. New England shells: obverse and reverse aspects of shells whose dimensions are given in Table VII.

In conclusion, I wish to thank Mr. W. J. Clench, Curator of Mollusks in Harvard University, for his advice, criticism and kindness.

TABLE VII

New England shells: dimensions and proportions of shells illustrated in Fig. 10.

Locality	Length (*a*)	Breadth (*b*)	Spire to Lip (*c*)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
	mm.	*mm.*	*mm.*			
10. Westerly	12.4	8.3	11.3	1.494	1.097	1.361
11. Westerly	13.2	9.0	12.3	1.467	1.074	1.367
12. Westerly	11.2	7.9	10.5	1.418	1.067	1.329
13. South Cohasset	10.4	7.0	9.8	1.486	1.059	1.400
14. South Cohasset	10.3	7.0	9.9	1.471	1.040	1.414
15. South Cohasset	9.3	6.0	9.0	1.560	1.033	1.500
16. Briar Neck	11.8	8.0	10.8	1.475	1.092	1.350
17. Briar Neck	13.0	9.0	12.4	1.444	1.048	1.378
18. Briar Neck	14.2	9.4	14.0	1.511	1.014	1.489
19. Rye Beach	12.8	8.7	11.7	1.471	1.094	1.345
20. Rye Beach	11.7	8.1	11.1	1.444	1.064	1.371
21. Rye Beach	13.0	8.5	12.4	1.530	1.048	1.459
22. Broad Cove	12.9	8.9	11.9	1.450	1.084	1.337
23. Broad Cove	11.7	8.0	11.7	1.462	1.000	1.462
24. Broad Cove	11.5	7.7	11.9	1.494	0.966	1.545
25. Port Clyde	12.4	8.5	11.5	1.459	1.078	1.353
26. Port Clyde	11.9	7.9	11.2	1.506	1.063	1.418
27. Port Clyde	12.4	8.7	12.6	1.425	0.984	1.448
28. Tenants Harbour	10.0	7.0	9.7	1.429	1.031	1.386
29. Tenants Harbour	10.2	6.9	10.2	1.479	1.000	1.479
30. Tenants Harbour	9.3	6.2	9.7	1.500	0.959	1.564
31. Isle au Haut	10.5	6.8	9.8	1.544	1.072	1.441
32. Isle au Haut	10.5	6.8	10.3	1.544	1.020	1.515
33. Isle au Haut	11.5	7.1	11.7	1.619	0.983	1.647

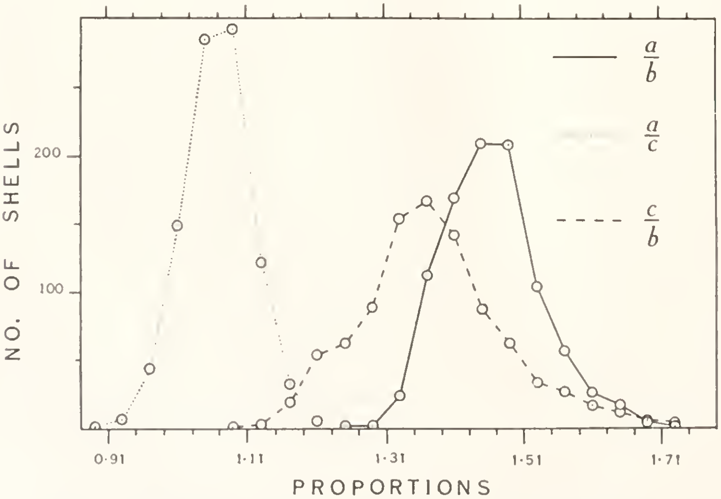

FIG. 11. Nine hundred and thirty-three shells from eleven localities on both sides of the Atlantic: numbers of shells of different proportions (See Table VIII).

TABLE VIII

Nine hundred and thirty-three shells from eleven localities on both sides of the Atlantic: numbers of shells of different proportions.

Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.87 to 0.91		1	
0.91 to 0.95		7	
0.95 to 0.99		44	
0.99 to 1.03		148	
1.03 to 1.07		284	
1.07 to 1.11		291	1
1.11 to 1.15		121	3
1.15 to 1.19		32	19
1.19 to 1.23		5	53
1.23 to 1.27	2		62
1.27 to 1.31	2	1	88
1.31 to 1.35	24		154
1.35 to 1.39	112		167
1.39 to 1.43	169		141
1.43 to 1.47	209		87
1.47 to 1.51	208		62
1.51 to 1.55	104		33
1.55 to 1.59	56		26
1.59 to 1.63	26		16
1.63 to 1.67	17		11
1.67 to 1.71	4		5
1.71 to 1.75	1		4
1.75 to 1.79			1

SUMMARY

1. *Littorina obtusata* from Norway, from the Plymouth district in England, and from Rhode Island are so alike that they cannot be separated.

2. *L. obtusata* from Rhode Island are fairly unlike those from Maine, but the examination of forms from intermediate localities establishes a continuous series up the New England coast. The range of variation remains roughly constant.

3. Further confirmation of the unity of *L. obtusata* from this wide geographical range is found by adding together the data from all the 933 shells examined. Their proportions follow almost perfect monomodal frequency curves.

4. The names *L. littoralis* (L.) and *L. palliata* (Say) must therefore go into synonymy under *L. obtusata* (L.), since it is shown that there is no division possible between forms to which these names have been given. The name *L. rudis* (Maton) must be put into synonymy,

under *L. saxatilis* (Olivi), as shown by Dautzenburg and Fischer (1912). The first definition of the genus *Littorina* is given by Férussac (1822) on p. xi of his Tabl. Syst. gén de l'emb. des moll.

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INHERITED VARIATION ARISING DURING VEGETATIVE REPRODUCTION IN PARAMECIUM AURELIA[1]

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INTRODUCTION

Jennings (1908), and Jennings and Hargitt (1910) have shown that populations of *Paramecium* which are produced from a single individual are uniform in size, form and fission rate. Other investigators have since confirmed these findings in other genera of Protozoa. Increased variation and the production of different stocks have been found to be produced by conjugation by Jennings (1913), Raffel (1930), Jennings, Raffel, Lynch, and Sonneborn (1932). Other investigators, Middleton (1918), Mast (1917), and Jollos (1921), have found inherited variation arising during vegetative reproduction which they attributed to the action of environmental agents. However, most investigators agree that when a clone of *Paramecium* is cultivated under uniform conditions it is usually decidedly uniform. An excellent review of the literature has recently been given by Jennings (1929).

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[1] The present contribution is one of a series of studies on the genetics of conjugation and reproduction in Protozoa, in progress by H. S. Jennings, his associates, and students. The author wishes to record his indebtedness to Professor Jennings for his interest and advice throughout the course of the work.

[2] Fellow of the National Research Council.

This paper presents the results of a study of a clone which sporadically produced branches, or lines, which differed markedly from the original type. These lines, which will be described in detail later, differed in many respects from the unaltered lines and these differences persisted for long periods under identical conditions. None of the altered lines was ever observed to revert to the original type although some were cultivated for nearly 100 generations.

Materials and Methods

The organisms used in this investigation were members of a clone of *Paramecium aurelia* descended by fission from a single ex-conjugant (known as 128*a*) obtained July 15, 1930, in the course of another investigation.

The methods of cultivation employed were the same as those used in the author's (Raffel, 1930) earlier work except that it was found necessary to add a small quantity of the bacteria to the fresh culture fluid each day. The culture medium consisted of a fluid containing KNO_3, 0.5 gram; K_2HPO_4, 0.06 gram; $MgSO_4.7H_2O$, 0.02 gram; $FeCl_3$, 0.001 gram; H_2O, 1 liter. To this solution cultures of an alga, *Stichococcus bacillaris,* and a bacterium, usually *Achromobacter candicans,* were added. The paramecia were cultivated in this suspension under bacteriological conditions.

Description of the Altered and Unaltered Lines of the Clone

1. *Origin.*

The clone 128*a* differed markedly from the other clones isolated from a group of individuals undergoing conjugation July 15, 1930. It was found to have a characteristic form and size quite diverse from the form and size of the other clones which were studied. It also differed from most of the other clones in its slow rate of reproduction. For these reasons it was selected as one of the few clones to which was devoted further intensive study. Later study has shown that it also differed from most of the other clones in its tendency to give off branches quite different from the main clone and in its ability to withstand adverse environmental conditions.

On August 4, 1930, twenty-two days after this clone was isolated, two of the twenty-four lines which were being cultivated became radically altered in ways to be described below. Three other lines became altered in a similar manner during the next three days. These five lines were discarded on August 15. During the five days following August 29, seven more lines became altered in a similar manner. These were cultivated until September 18, when they were discarded. No other altered lines appeared until early in November, but from that time until June of the following year, they were produced very frequently.

2. *Comparison of the unaltered and altered lines.*

The typical individuals of this clone, hereafter referred to as the unaltered lines, differed from the altered lines in the following ways:

(a) Size. One of the most striking differences between the two groups was the difference in size. This difference is well illustrated by Fig. 1, which shows typical specimens of each group under identical conditions. As can be seen from this figure the individuals of the altered lines were more than twice as long as those of the unaltered lines. This marked size difference was a matter of continuous daily observation.

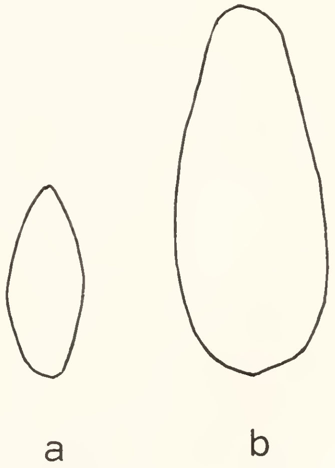

FIG. 1. Camera lucida drawings of typical individuals of (*a*) unaltered and (*b*) altered lines.

(b) Form. The two branches of clone 128*a* differed markedly in form. The unaltered individuals of this clone were thick in the central region and tapered to points at each end. They were quite pale and usually had a dark food vacuole in the anterior tip. The altered individuals were, on the other hand, of the typical *P. aurelia* form, *i.e.*, somewhat pointed anteriorly, rounded posteriorly with their greatest breadth about two-thirds from the anterior end. Usually they had many food vacuoles distributed chiefly in the posterior part of the organism. These differences in form as well as the size differences are shown clearly in Fig. 1. This figure shows typical adult individuals of both kinds living under identical conditions and drawn at the same time. The differences in form and also the differences in size persisted even during the periods of depression when the fission rates of the two groups approached one another. At these times the two groups could be readily distinguished by their appearance.

(c) Fission Rate. The altered lines differed from the unaltered lines greatly in their rates of reproduction. The clone originally was a very slowly dividing one with a mean fission rate much below one division per day. The altered lines, on the other hand, divided very vigorously and some of them averaged well over two fissions per day for periods exceeding two weeks. This difference in fission rates is illustrated in Fig. 2. The solid line in Fig. 2 shows the mean daily fission rates for the original clone from July 15, 1930 to May 17, 1931, plotted in approximately five-day periods. The dotted lines represent the average daily fission rates of six different groups of altered lines which

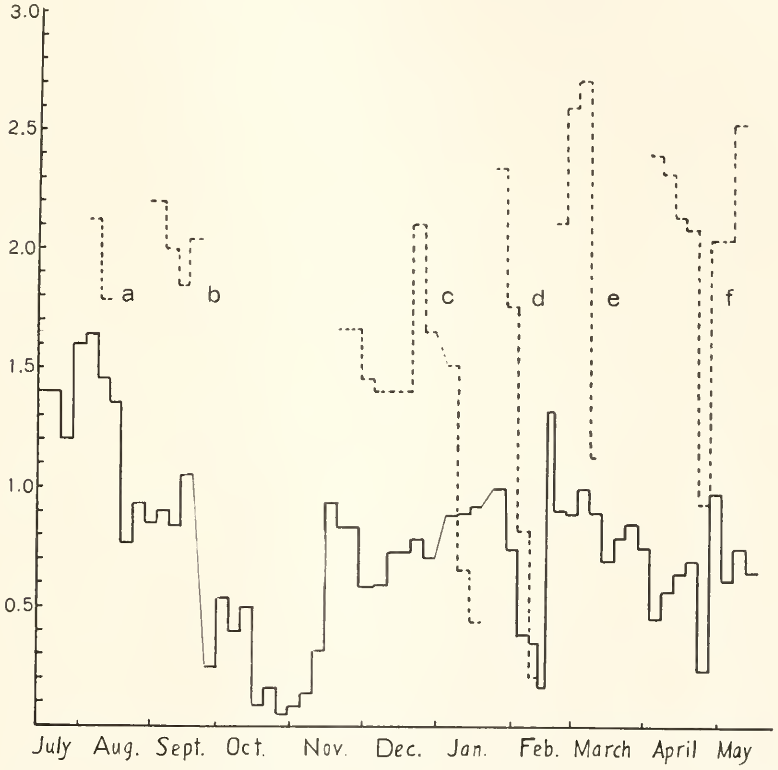

FIG. 2. Average daily fissions of unaltered (solid line) and of altered (dotted lines) lines plotted in periods of approximately five days each.

were studied. These are also plotted for the most part in five-day periods. From this figure it is evident that the altered lines differ markedly from the unaltered lines in their rates of reproduction.

(d) Resistance. The altered lines of clone 128*a* were much less resistant to unfavorable conditions than were the unaltered lines. This can be readily observed from Fig. 2 which shows that in nearly every period of depression the altered lines suffered a decrease in fission rate earlier than the unaltered lines, and that the extent of the depression was greater in the altered lines. For example, group (*c*) Fig. 2 declined in fission rate from 1.26 to 0.43 fissions per day between January 10 and January 20, while the unaltered lines showed an actual increase from 0.88 to 0.93 fissions per day during the same period. Similar though not so great differences can be found by comparing groups (*d*), (*e*) and (*f*) with the unaltered lines which were cultivated simultaneously with them.

We have seen then that the clone 128*a* produced branches during vegetative reproduction which differ markedly in size, form, fission rate, and resistance from the unaltered lines. The differences in size and form persist even during periods of depression during which the fission rates of the two groups were similar. These differences are as great as any found by Jennings, Raffel, Lynch, and Sonneborn (1932) in their extensive study of diversities between biotypes produced by conjugation. The altered lines persisted for long periods and none has ever been observed to revert to the original condition although many thousands of individuals have been examined.

EXPERIMENTS

1. *Regularity in the production of altered lines.*

The remaining parts of this paper deal with experiments designed to discover whether any regularity was apparent in the production of these altered lines, and whether after conjugation these unlike branches of the same clone would produce similar or dissimilar populations. In order to learn more about the occurrence of the altered individuals and to ascertain whether there is any regularity in their production, the following experiments were performed:

From November 3rd to 19th the progeny of one individual of the clone was expanded to form 336 lines of descent whose relationships were known. During this period some of the lines became altered. These, because of their faster rate of reproduction, gave rise to the greater part of this population. After the desired number had been obtained, the organisms were cultivated under identical conditions for ten days. During this period the lines which were altered at the beginning passed through from 1–21 generations (all but one of the 226 such lines which lived until November 29 passed through from 11–21 generations), while the unaltered lines passed through from 4–9 generations. As is shown in Fig. 2 (*c*), the mean daily fission rate for the altered lines is 1.67 as compared with 0.83 fissions per day for the unaltered lines.

On November 29 all except one of each of the altered and unaltered lines were discarded. Then each of these was expanded to a population of 168 individuals. These two populations were then cultivated for ten days from December 11–21, during which time they showed great diversity in their size, forms and fission rates (*see* Table I). Although the altered lines were much depressed at this time because of unfavorable environmental conditions or endomixis, the population derived from the altered individual produced no individuals which were like the unaltered lines.

TABLE I

Distribution of total fissions December 12–21, 1930, of populations derived from (a) a single individual which manifested the altered set of characteristics on November 18, and (b) from an individual of the original type.

Fissions	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Mean
Altered lines	1			1		1	3	3	7	7	2	3	9	12	12	24	13	10	4	13.1
Unaltered lines	0	1	5	10	12	17	18	8	6	5	5	5	6	5	4	3	2			7.2

Figure 3 is a genealogical chart showing the relationships of the lines descended from the unaltered individual, with their numbers of fissions during the ten days following December 11. These lines were all of the original type on December 11; but, as is shown, many of them, represented by the filled-in circles on Fig. 3, became altered subsequently.

From the data given in Fig. 3, an attempt was made to ascertain whether or not there is any regularity or rule in the production of altered stocks. That is, if a given line is altered, are the lines which are separated from it by fewest fissions more likely to be altered than lines separated by more fissions or not; or is any other rule to be found which would describe the production of the altered lines? To investigate this question the coefficients of correlation were obtained for the total number of fissions between December 12 and December 21, of lines represented on Fig. 3 which were separated on December 11 by one, two and three fissions. The lines which were separated by but one fission at this time showed a correlation of 0.56 ± 0.06; those separated by two fissions a correlation of 0.23 ± 0.12; while those separated by three fis-

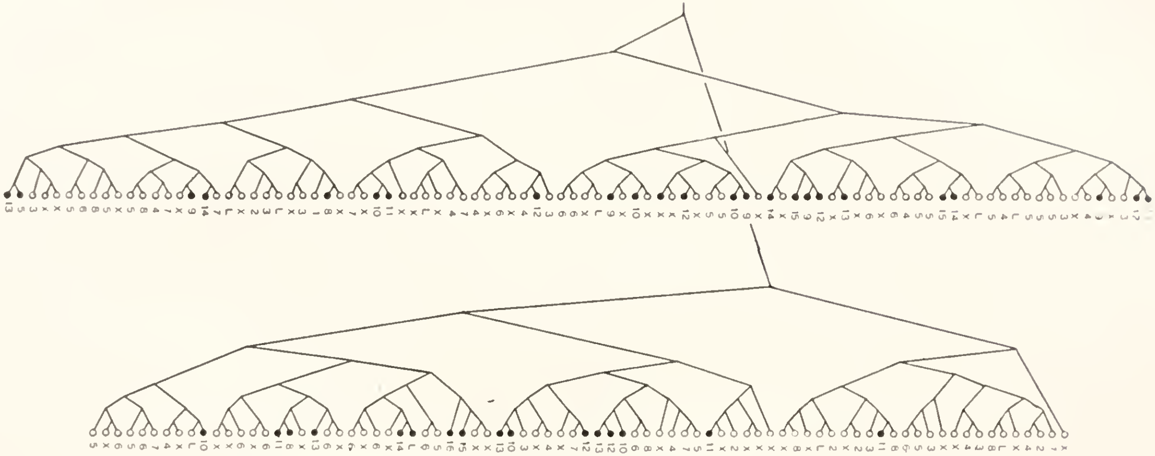
13 5 3 x x 5 6 8 5 x 5 8 4 7 x 9 14 7 L x 2 3 L x 3 1 8 x 7 x 10 11 x x L x 4 7 4 x 6 x 4 12 3 6 6 x L 9 x 10 x x x 12 x 5 5 10 9 x 14 x 15 3 12 x 13 x 6 x 6 4 5 5 15 14 x L 5 4 L 5 5 5 3 x 4 3 x 3 12
5 x 6 5 6 7 4 x L 10 x x 6 x 6 11 8 x 13 6 x 6 x 6 x 14 L 6 5 16 15 x x 13 10 3 x 4 x 7 12 13 12 10 6 8 x 4 7 5 11 x 2 x x x x 8 x L 2 x 2 3 11 8 6 5 3 x x 4 3 8 L x 4 2 7 x

Fig. 3. Genealogical chart showing the relationships of the lines derived from an unaltered individual between November 29 and December 11, 1930, with the number of fissions of each line from December 12–21 inclusive. The lines were all unaltered on December 11. Those which remained unaltered are represented by open circles, those which became altered by solid circles.

X indicates that the line died before December 21.

L indicates that the line was lost.

sions gave a coefficient of correlation of 0.48 ± 0.06. Thus there is apparently no relation between the number of fissions by which lines are separated and the fate of the lines. The decrease in the coefficients of correlation of the lines separated by two fissions as compared to those which are separated by only one appears to be meaningless when we find that those separated by three fissions do not differ significantly from those separated by only one fission. A careful study of the records, however, shows that when two lines are derived from a common ancestor, one or two generations before one of them becomes altered the other usually becomes altered in a similar manner at nearly the same time. This occurred frequently throughout the entire six months that this subject has been intensively investigated.

The experiments and observations which were made with the object of discovering any regularity or rule in the production of altered lines did not give any evidence of any such regularity. They did show, however, that if a line becomes altered, lines which were derived from the same individual not more than two generations previously generally became altered at the same time or at nearly the same time.

2. *Effect of conjugation in the diverse lines.*

In another paper Jennings, Raffel, Lynch, and Sonneborn have shown that when two diverse clones conjugated they produced two very diverse populations. In the two clones intensively studied, although each population showed great variation in fission rates, the mean fission rate of each was not very different from the fission rate of the clone which produced it. In the particular cases which they studied the mean fission rates of the two populations differed in the same direction and to approximately the same extent from the mean fission rates of the clones from which they came. In the present investigation we have two races which differ more than the two clones studied by Jennings, Raffel, Lynch, and Sonneborn. These two races are, however, not different clones but are branches of the same clone; that is, they are descended by vegetative reproduction from a common ancestor. Will these two very diverse races also produce by conjugation populations which are on the whole diverse, or will they, unlike diverse clones, produce populations which are similar? In order to answer this question typical lines of the two races of this clone were chosen and expanded in isolation drop cultures until large numbers of both types were available. Then conjugation was induced in both branches of the clone and 105 pairs of conjugants and 96 non-conjugants were taken in each group. In the case of the slowly reproducing unaltered lines care was taken to choose among the non-conjugants some split pairs and others which were beginning to conjugate. These four sets of organisms were then cultivated side by side for ten days after the last ex-conjugants were isolated.

The results of this experiment are shown in Figs. 4 and 5. Figure 4 shows the course of the mean daily fission rate of the two groups of non-conjugants; and Fig. 5 shows the same for the ex-conjugants. As can be seen, the non-conjugants of the unaltered lines continued to reproduce at the rate of approximately 0.9 fissions per day, while the ex-conjugants from this group reproduced much more rapidly, reaching at the end of the experiment a rate of 1.6 fissions per day. This progressive increase in fission rate was due entirely to the dying of the many abnormal lines which were produced by conjugation. Ninety-three non-viable lines were produced. Only 10 of 210, or 4.8 per cent, of the ex-conjugant lines resembled the parent race in fission rate. Among the 96 lines of the non-conjugants, 29 became altered and 24 lines died out during this period.

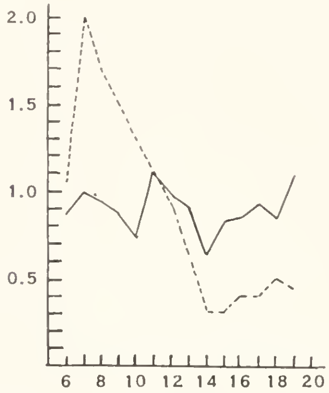

FIG. 4

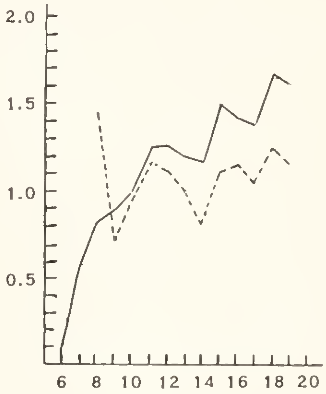

FIG. 5

FIG. 4. Average fissions on successive days of non-conjugant lines of the unaltered (solid line) and altered (dotted line) groups.

FIG. 5. Average fissions on successive days of the ex-conjugant lines of the unaltered (solid line) and the altered (dotted line) groups.

The results obtained from the altered lines were quite different. The non-conjugants at the beginning were very healthy and vigorous, reproducing at the rate of two fissions per day the second day after conjugation and nearly as rapidly the next day. From then on they declined rapidly in vigor—the fission rate falling to between 0.3 and 0.5 fission per day, accompanied by a great increase in mortality. The ex-conjugants, which were also very vigorous at first, with a fission rate of 1.4 for the first day, declined to approximately one fission per day in spite of the death of the 90 non-viable lines which had been produced by conjugation.

Because of the depressed conditions of the organisms during this experiment it seemed advisable to repeat it under more favorable conditions. Great difficulty was experienced in trying to obtain the conjugants from the unaltered lines as these were constantly producing organisms of the altered type, which in a short time would greatly outnumber the type which the culture originally contained. Finally, in order to know which type of organisms was conjugating in the cultures of the original group, split pairs were taken at the same time as the conjugants. Thirty-three per cent of all of the organisms obtained from split pairs were of the original type. However, no pair among the split pairs was composed of two organisms of this type. It is very probable that this was due in a great measure to the subsequent alteration of one member of the pairs, as altered lines were being produced rapidly at this time.

TABLE II

Distribution of fissions during the first five days after conjugation of ex-conjugants derived from (a) altered lines and (b) unaltered lines of clone 128*a*.

Fissions..........	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	No. of lines	Mean
Altered lines......	4	0	1	1	2	3	3	9	10	14	17	10	24	33	19	1	151	10.5
Unaltered lines....	0	4	2	3	4	11	6	6	8	8	19	18	38	46	24	19	216	10.9

The results of the conjugations in the two branches were as follows: The unaltered lines gave rise to a population very different from themselves. The mean fission rate of 2.2 fissions per day is much greater than the 0.62 fission per day of the non-conjugants which lived through the same period. The ex-conjugants were somewhat less viable than the non-conjugants of this group; 22.3 per cent of the former died during this period as compared to 18.9 per cent of the non-conjugants. Only 11 of the 278 ex-conjugant lines or 4 per cent had the shape and size of the unaltered lines.

On the other hand, the altered lines gave a quite different result. The ex-conjugant lines were much less viable than the non-conjugants; 34.7 per cent of the lines derived from ex-conjugants died, while there was no mortality in the non-conjugants. The mean fission rate of the ex-conjugants was 2.1 fissions per day as compared with 2.3 for the non-conjugants. No single individual among the ex-conjugant lines obtained from the altered group had the size or form of the unaltered lines. A comparison of the two groups of ex-conjugants with respect to their fission rates is given in Table II. This table shows the great similarity between the fission rates of the two populations which have the same mode and means which differ very slightly.

These results differ markedly from those obtained by Jennings, Raffel, Lynch, and Sonneborn, on the effects of conjugation in their two diverse clones. The two populations produced after conjugation by the two diverse branches of the same clone, in the investigation presented here, are nearly identical with respect to their fission rates. The mean fission rates of both populations are nearly equal to the mean fission rate of the altered population. In the case of the altered lines this result is in accordance with the results obtained by earlier workers. Jennings (1913) and Raffel (1930) found that conjugation within a clone results in a population showing much variation, with a mean fission rate slightly lower than that of the parent clone, and an increase in mortality. In the case of the unaltered lines of this clone the effect of conjugation is to produce a population quite different from the non-conjugant population. The mean fission rate is increased to more than three times the mean fission rate of the non-conjugants and the size and shape of 96 per cent of the ex-conjugants is altered. Thus the unaltered lines of clone 128*a* are affected by conjugation in a way quite unlike most of the clones which have previously been described.

It is obvious that the two very diverse branches of clone 128*a* are affected quite differently by conjugation. Instead of giving rise to two diverse populations as did the two diverse clones investigated by Jennings, Raffel, Lynch, and Sonneborn, they produced, after conjugation, populations nearly identical in fission rates and appearance. This indicates certainly that the inherited diversity which appeared during vegetative reproduction in this clone differs in some way from the diversities between the clones studied by Jennings, Raffel, Lynch, and Sonneborn.

Discussion and Conclusions

The inherited variations which arose in this clone during vegetative reproduction differ markedly from those which are produced by conjugation. The altered lines which were produced were similar to one another in all respects and they were quite different from the original type, while conjugation produces from a single clone a large number of clones which differ much or slightly from one another so that a more or less continuous series is produced. The differences between the two branches of this clone are to a great degree eliminated by conjugation while diverse clones produced by conjugation usually yield after subsequent conjugations populations which are similar to themselves in their fission rates and other characteristics. This suggests that the basis of heritable variation found in this clone is different from that of the variation produced by conjugation.

The increase of variation after conjugation and the cytological details of that process have led to the conclusion that conjugation involves biparental inheritance and that the increased variation produced by conjugation is brought about by a recombination of genetic factors. The inherited variation reported in this paper, however, does not seem to arise from such recombinations. It differs from the variations produced in that manner as set forth above; and in order to ascribe its origin to such recombinations it would be necessary to postulate some such process for this particular clone only. Therefore, it seems that recombinations must be dismissed as a possible explanation of the origin of this variation.

That the genetic constitution of the original clone must contain a number of heterozygous pairs of genes is evident from the results of conjugation between members of the unaltered branch. In order that a particular clone should produce, when inbred, a population only 4 per cent of which resembles the parent, it is necessary that the clone should be heterozygous for four or five genes. Therefore, we can assume that a heterozygous condition of four or five pairs of genes is necessary for the production of the unaltered type of this clone. A mutation then of any of eight or ten genes would produce an altered line in this clone. It is possible, however, that the observed altered lines could only be produced by the mutation of any of the four or five " mutant " genes to the " normal " condition (as a mutation of the " normal " gene might be lethal when homozygous). The evidence leads to the conclusion that the production of altered lines in this clone is probably caused by the mutation of one of the members of the four or five pairs of heterozygous genes.

This conclusion explains all of the phenomena observed. There is no regularity in the production of the altered line—no regularity would be expected of gene mutations. The altered lines never produce the original type after conjugation—as it is not heterozygous for all five pairs, it cannot produce progeny which are heterozygous for all five pairs.

The only question which this conclusion raises is the frequency of gene mutation in *Paramecium*. This is to be the subject of a subsequent paper, but it might be said here that this explanation of the questions raised by this investigation throws some light on other questions. Jennings, Raffel, Lynch, and Sonneborn found that clones differ in their uniformity. Others have found variation between clones in mortality, etc. If gene mutations are comparatively frequent in *Paramecium*, it can account for the cases of inherited variation reported by other workers as well as the unexplained mortality that occurs in all isolation culture work. It, furthermore, can explain the differences in mortality which are observed in different clones. The only alternative to this explanation is that the original clone contained a detachable translocation which was lost in the production of the altered branches. The former explanation seems preferable because it explains the numerical relationships found in this investigation as well as the other phenomena which have been mentioned; and also because much evidence has subsequently been obtained which indicates frequent mutations in *Paramecium*. A more detailed treatment of these questions will be presented in the near future.

SUMMARY

The clone of *Paramecium aurelia* studied in this investigation produces branches which differ from the original clone in many respects. They are larger, have a different form, reproduce at a greater rate, and are less resistant to unfavorable conditions than the original type. These branches consistently manifest their diverse characteristics and none has ever been known to revert to the original type in any of the cases which were studied. No rule or regularity was found in the production of the altered lines except that when a line became altered, lines which were separated from it by only one or two fissions usually became altered at the same time or nearly the same time. Unlike diverse clones, these diverse races of the same clone give after conjugation populations which are on the whole very similar. The unaltered type gave after conjugation only a very small proportion (4 per cent) of lines which were similar in size, form and fission rate to the parent clone.

From these observations and experimental results the conclusion is drawn that the variation is produced by mutations of one member of the four or five heterozygous pairs of genes which are necessary to produce the normal type of this clone.

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RACIAL DIFFERENCES IN THE EARLY PHYSIOLOGICAL EFFECTS OF CONJUGATION IN PARAMECIUM AURELIA

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I. Introduction

The Problems and Their Present Status

The question of the effect of conjugation has usually been proposed in the form: "What is the effect of conjugation in the ciliate Protozoa?" The possibility that different species might show different effects of conjugation has been suggested, but the possibility that similar differences might be found between different lines of descent within a single species has received but scant attention. Over forty years ago Maupas (1888) attempted to demonstrate that conjugation between unrelated (or distantly related) individuals was fertile, but that conjugation between close relatives was not. Calkins (1919 and 1920), working on *Uroleptus mobilis*, and Woodruff and Spencer (1924), working on *Spathidium spathula*, found that conjugation in much depressed lines did not usually bring the ex-conjugants to so high a reproductive rate as did conjugation in the less depressed lines. In other words, there was a positive correlation between the mean fission rates of the non-conjugants and the mean fission rates of the derived ex-conjugants. MacDougall (1931) has shown that morphologically different races of *Chilodon uncinatus* yield similar morphologically different groups of ex-conjugants. Jennings *et al.* (1932) have shown that in *Paramecium aurelia* two ex-conjugant clones which differ markedly in mean fission rate yield groups of ex-conjugants which differ similarly in mean fission rate. Raffel (1932), on the other hand, working on a single ex-conjugant clone of the same stock of *Paramecium aurelia* employed by Jennings *et al.,* found that two sub-clones with considerably diverse fission rates which arose from this single clone during vegetative reproduction, yielded groups of ex-conjugants that were not significantly different. (It needs scarcely to be recalled that in the work of Jennings *et al.,* and of Raffel, great differences were found within each group of ex-conjugants; the values compared are merely mean values. See the original papers for details.)

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[1] The present contribution is one of a series of studies on the genetics of conjugation and reproduction in Protozoa, in progress by H. S. Jennings, his associates, and students. The authors wish to record their indebtedness to Professor Jennings for his interest and advice throughout the course of the work. This investigation was aided by a grant from the Bache Fund.

These results of Woodruff and Spencer, MacDougall, and Jennings *et al.* strongly support the idea that the effects of conjugation may differ in different lines of descent within the same species. Only in the work of MacDougall, however, has there been repeated demonstration in the same races, of the same differences in the effects of conjugation. In her work, the differences studied were morphological. There is on record no thorough study of characteristic racial differences in the effects of conjugation on fission rate, variability or mortality. These physiological characteristics are the ones most intimately related to current theories of conjugation. Studies on characteristics such as these are required in order to determine whether the differences in results, and in theories constructed from these results, as set forth by different investigators, are due partly or entirely, not to specific differences, but merely to racial differences in the stocks they studied. Might conjugation result in rejuvenescence in some races of a species and not in others? Might conjugation result in great increase in variation in some races of a species and in little or no increase in variation in others? Might conjugation result in great mortality in some races and in little or no mortality in others?

If conjugation in Protozoa is fundamentally similar to zygote formation in higher organisms, then, as suggested by Jennings (1929), conjugation should result in similar recombinations of chromosomes and genes. The present state of knowledge of the genetics of the Protozoa, unlike the state of knowledge of the genetics of higher organisms, does not justify the assumption of genes; but the works of Jennings (1911, 1913), Jennings and Lashley (1913, 1913*a*), Pascher (1916), Raffel (1930), and Jennings *et al.* (1932) demonstrate that conjugation in Protozoa is a process resulting in biparental inheritance and in the formation of many new hereditary types. If the nuclear recombinations in the conjugation of Protozoa further resemble the nuclear phenomena in gamete and zygote formation of higher organisms, racial differences in the effects of conjugation might be predicted. Races which differed in the number of heterozygous "gene" pairs (or whatever the corresponding determiners in Protozoa may be called) would differ in the amount of variation produced by conjugation. Races which differed in the number of heterozygous lethals would differ in the mortality produced by conjugation. Races which differed in the genes determining fission rate would yield conjugants which differed in these respects; thus some races might be rejuvenated by new, favorable, chromosomal recombinations, other races depressed by new, unfavorable chromosomal recombinations.

The importance of the issue for current theories of conjugation, the seeming likelihood of obtaining a result throwing a light on the possible analogy between conjugation in Protozoa and zygote formation in higher organisms, and the fact that encouraging results had already been obtained both in our own work and in the work of others, induced us to study intensively this matter of characteristic physiological differences between races, in the effects of conjugation.

Plan of Investigation

The present investigation begins at the point reached by the results of the investigation of Pascher and of Jennings and his collaborators, mentioned above, and goes on to ask the following questions:

1. When the diverse clones produced by conjugation within a single clone are allowed to conjugate, each within itself, do they all produce similar sets of new ex-conjugant clones, or do the results of conjugation differ in the diverse parent clones? That is, does conjugation produce the same result in slowly multiplying clones as in those that multiply rapidly? Does conjugation produce similar results in clones differing in other respects?

2. If different results are produced, is there a correlation between the characteristics of the parent clones and those of derived ex-conjugant clones? For example, does a slowly multiplying clone produce a set of ex-conjugants that multiply on the average less rapidly than the set produced by a rapidly multiplying clone?

3. Do successive conjugations among the members of a single clone yield similar sets of ex-conjugant clones in each case? Or do different conjugations within the same clone yield diverse results?

4. Does the mean fission rate of the ex-conjugant clones always bear the same relation to the fission rate of the respective parent clone? Or may conjugation in some clones result in an increased fission rate, in other clones in a decreased fission rate?

To answer these questions, the following were studied (compare the diagram of Fig. 1):

A. Comparison of the effects of successive conjugations in the same clone. In the clone employed (designated 247*a*), part of the individuals were allowed to conjugate, while others were not; the two sets were then compared. Later, another set were allowed to conjugate, and these again compared with the non-conjugants. This was repeated for a third time.

B. Comparison of the effects of simultaneous conjugations in six different ex-conjugant clones, which had similar vegetative characteristics.

C. Comparison of the effects of repeated simultaneous conjugations in two of the ex-conjugant clones that had shown diverse results in the first comparison (*B*). In all, four successive simultaneous conjugations of the two stocks were compared.

D. Comparison of the effects of conjugation between the above two clones, with the effects of conjugation within each of these clones. This part of the work is but briefly reported here, as the matter is of such novelty and importance as to warrant treatment in a later separate publication, in which will be given also the results of other crosses that have been made.

In giving the results of these four sets of experiments, it will be observed that the data are mainly for the early effects of conjugation: for those manifested within the first two or three weeks after conjugation. During this early period occur many of the most characteristic effects of conjugation, such as the dying out of weak races. As to the relation between the earlier and later effects of conjugation, the present paper makes no assumptions. A comprehensive study of conjugation requires a knowledge of both its earlier and its later effects. It is planned to have later contributions in this series of investigations deal explicitly with the later results of conjugation, as compared with the earlier ones.

II. Materials and Methods

Throughout the course of the investigation, the paramecia were cultured in isolation, one paramecium to one drop of culture fluid. The culture drops were placed on double-concavity, hollow-ground slides. These slides were kept together in groups of twelve on a glass plate raised by glass supports above the bottom of a 10-inch inverted Petri dish sealed with distilled water. These chambers of isolation cultures were examined daily, at which time one animal from each culture drop was transferred to a fresh drop of culture fluid on another slide.

At different times during the course of the work three different methods of preparing the culture fluid were employed; these may be referred to as methods *A, B*, and *C*. Method *A* is the method described by Jennings *et al.* (1932). This consists of boiling 30 flakes of Quaker Rolled White Oats in 100 cc. spring water for 3 minutes; allowing this to stand five minutes; filtering into 250 cc. Erlenmeyer flask; letting filter and flask stand for 24 hours; then inoculating filtrate (now rich in bacteria from air and glassware) with the alga *Stichococcus bacillaris* (grown on agar slants). Method *B* is the method described by Raffel (1930), except that precautions designed to exclude foreign bacteria were not employed. Raffel's synthetic fluid was inoculated, just before using, with the bacterium *Achromobacter candicans* (grown on agar slants) and the alga *Stichococcus bacillaris* (also grown on agar slants). Culture method *C* is merely a slight modification of method *A*. It differs from method *A* in two particulars: (1) The first part of the filtrate, which comes through the filter rapidly in the first five minutes, is rejected; (2) The 24-hour-old infusion is boiled and cooled just before inoculating with *Stichococcus*. These modifications of method *A* were found necessary at certain times to keep within satisfactory limits the quantity of bacteria in the culture fluid.

The paramecia used in the present study were all descendants of a single ex-conjugant, known as 247*a*, from Raffel's intra-clonal conjugation of July 15, 1931, reported by Jennings *et al.* (1932). All individuals descended from 247*a* by vegetative reproduction without the intervention of conjugation are known as the clone 247*a*. This clone was kept under observation throughout the entire course of the experiments from October 1, 1930, till April 1, 1931. In Fig. 1, which gives a graphic account of the course of the experiments, the continuous base line represents the clone 247*a*. On October 1, October 16, and December 9, parts of the clone 247*a* were allowed to conjugate; this is represented by the oblique lines leading to *C, D*, and *E*. The group of conjugants obtained October 1 was called the *C* group; it consisted of 58 pairs. After separation of the members of the pairs each gave rise to a single line of vegetative descent; some of these lines of descent are represented in Fig. 1, by straight lines parallel to the 247*a* line. The *D* group beginning October 16, consisting of 100 lines of descent from 50 pairs of conjugants, and the *E* group beginning December 9, consisting of 194 lines of descent from 97 pairs of conjugants, are represented in a similar way.

From this *E* group were selected for further examination lines of descent from six of the ex-conjugants. These as shown in Fig. 1, were ex-conjugant clones *E*40*a*, *E*41*a*, *E*46*b*, *E*80*b*, *E*81*a*, and *E*85*b*. On January 12–14, conjugation was simultaneously induced within each of these six ex-conjugant clones. These conjugations, known as *F, G, L,* *K, J,* and *H,* respectively, each consisted of 48 pairs of conjugants. Two of the six clones, namely *E*40*a* and *E*81*a*, were kept for further comparisons. In these two clones, simultaneous conjugations were induced on February 9–12, February 15–18, and March 11–16. These simultaneous conjugations are designated *P* and *M*, P_1 and M_1, and *S* and *T*, respectively. At the same time as the conjugations *S* and *T* were taking place within the races *E*40*a* and *E*81*a*, conjugation *X* was also taking place. This was a cross-conjugation between representatives of *E*40*a* and *E*81*a*.

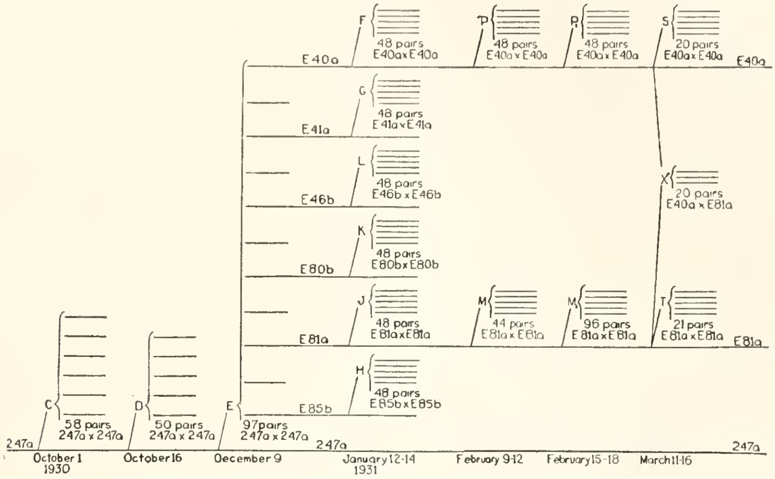
F
48 pairs
E40a x E40a
P
48 pairs
E40a x E40a
P1
48 pairs
E40a x E40a
S
20 pairs
E40a x E40a
E40a
E40a
G
48 pairs
E41a x E41a
E41a
L
48 pairs
E46b x E46b
E46b
X
20 pairs
E40a x E81a
K
48 pairs
E80b x E80b
E80b
J
48 pairs
E81a x E81a
M
44 pairs
E81a x E81a
M1
96 pairs
E81a x E81a
T
21 pairs
E81a x E81a
E81a
E81a
H
48 pairs
E85b x E85b
E85b
C
58 pairs
247a x 247a
D
50 pairs
247a x 247a
E
97 pairs
247a x 247a
247a
247a
247a
October 1
1930
October 16
December 9
January 12-14
1931
February 9-12
February 15-18
March 11-16

FIG. 1. Pedigree chart of the clones used in this investigation; all clones were derived, as shown, from the clone 247*a*. The horizontal lines represent single ex-conjugant clones (descendants of a single ex-conjugant) multiplying vegetatively without the intervention of conjugation. An oblique line branching off from a line representing a clone indicates that a part of this clone was allowed to conjugate. Each group of conjugants is designated by a single capital letter to which an oblique line leads. The brackets following this letter enclose a set of horizontal lines representing some of the new ex-conjugant clones. Below these lines are given, in parentheses, the number of pairs of conjugants and the clone-names of the conjugating individuals.

In the course of the experiments, sixteen different sets of conjugants and their descendants were studied. These are listed, together with

TABLE I

General Information Concerning the 16 Conjugations Studied

Clones from which conjugant pairs were derived	Name of group of ex-conjugants	Dates of conjugation	Period observed	Number of pairs	No. of lines of each ex-conjugant	Total no. of lines	Temperature range in degrees centigrade	Culture method
247a × *247a*	*C*	Oct. 1–2 1930	Oct. 1–14	58	1	116	21°–23°	*B*
247a × *247a*	*D*	Oct. 16–20	Oct. 16–Nov. 2	50	1	100	Oct. 16–25: 21°–23° Oct. 26–29: 23°–25° Oct. 30–Nov. 2: 19°–22°	*B*
247a × *247a*	*E*	Dec. 9–15	Dec. 9–27	97	1	194	21°–23°	*A*
E40a × *E40a*	*F*	Jan. 12 1931	Jan. 12–25	48	1	96	17°–29°, but usually 21°–26°	*A*
E41a × *E41a*	*G*	Jan. 12–13	Jan. 12–25	48	1	96	ditto	*A*
E85b × *E85b*	*H*	Jan. 12–13	Jan. 12–25	48	1	96	ditto	*A*
E81a × *E81a*	*J*	Jan. 12–14	Jan. 12–25	48	1	96	ditto	*A*
E80b × *E80b*	*K*	Jan. 13–14	Jan. 13–25	48	1	96	ditto	*A*
E46b × *E46b*	*L*	Jan. 12–14	Jan. 12–25	48	1	96	ditto	*A*
E81a × *E81a*	*M*	Feb. 9–12	Feb. 9–22	44	1	88	23°–25°	*A*
E40a × *E40a*	*P*	Feb. 11	Feb. 11–14	48	1	96	23°–25°	*A*
E81a × *E81a*	M_1	Feb. 15–18	Feb. 15–28	96	1	192	23°–25°	*A*
E40a × *E40a*	P_1	Feb. 15–18	Feb. 15–28	48	1	96	23°–25°	*A*
E40a × *E40a*	*S*	Mar. 11–16	Mar. 11–Apr. 1	20	2	80	20.5°–28.5°, but usually 21°–26°	*C*
E81a × *E81a*	*T*	Mar. 11–16	Mar. 11–Apr. 1	21	2	84	ditto	*C*
E40a × *E81a*	*X*	Mar. 11–15	Mar. 11–Apr. 1	20	2	80	ditto	*C*
Total: *247a* × *247a*				205		410		
Total: *E40a* × *E40a*				164		348		
Total: *E81a* × *E81a*				209		439		

useful information about them, in Table I. There are given, in column 1, the names of the clones of the two conjugating individuals; in column 2, the designation given to each particular group of ex-conjugants; in column 3, the dates on which conjugation occurred; in column 4, the dates between which the ex-conjugants were studied; in column 5, the number of pairs of conjugants in each group; in column 6, the number of lines of descent from each ex-conjugant; in column 7, the total number of lines in each group; in column 8, the temperature range during each experiment; in column 9, the culture method employed.

Figure 1 and Table I are intended to give the general plan of the investigation and many of the pertinent details. At the beginning of each of the subsequent sections of the paper it may be helpful to refer both to Fig. 1 and to Table I.

III. Three Conjugations within the Clone *247a*

Most of the necessary information concerning the origin of clone *247a* and the details of the three conjugations within it have been given in the preceding sections. (See especially Fig. 1 and Table I.) With descendants of the 58 pairs of conjugants obtained on October 1–2, the twenty-four co-existing non-conjugant lines were compared during two successive five-day periods (December 18–22 and December 23–27).

Table II gives a detailed picture of the distribution of total numbers of fissions obtained in these experiments, and shows some relations that will be more precisely formulated by statistical methods in other tables. It is at once apparent that the range of fission rates of the ex-conjugants in every period exceeds that of the non-conjugants. Less obvious is the fact that the values for the ex-conjugants are less concentrated within the modal range than are the values for the non-conjugants. Calculations based on Table II show that on the average, only 73.3 per cent of the ex-conjugant values for five-day periods are concentrated in the three most frequent classes of values, whereas 93.1 per cent of the non-conjugant values are within their three most frequent classes.

Table II also shows clearly that the numbers of fissions in five days vary greatly from period to period in both groups. Taking the modal values as an illustration, the ex-conjugant mode drops from 11 fissions in the first period to 5 in the second period of the first experiment. The non-conjugant mode dropped from 10 to 6 during the same periods. These two periods are typical of the sort of changes that were observed in many experiments. At the time of such shifts of mode in one group, similar changes were observed in the reproductive rates of all the races being cultivated, so that it appeared practically certain that the change was due to some change in the conditions of cultivation.

TABLE II

Comparison of non-conjugants of clone 247a with three sets (C, D and E) of ex-conjugants derived from them at different times. Distribution of total number of fissions during five-day periods.

Five-day periods	Group	Total number of fissions														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	
Exp. 1. Oct. 5–9, 1930..	Ex-conjugant group *C*					1	1		1	4	8	19	35	21	5	Number of lines
	Non-conjugants of 247*a*										3	12	7			
Oct. 10–14, 1930......	Ex-conjugant group *C*	2	2	1	9	19	30	18	6	1	2					
	Non-conjugants of 247*a*		1		1		3	9	5							
Exp. 2. Oct. 24–28, 1930	Ex-conjugant group *D*	3	1	2		1	3	4	4	6	15	17	13	1		
	Non-conjugants of 247*a*			1						2	5	5	1			
Oct. 29–Nov. 2, 1930 ..	Ex-conjugant group *D*		1	1	8	17	10	3	8	2	1	1				
	Non-conjugants of 247*a*				1	5	5	2								
Exp. 3. Dec. 18–22, 1930	Ex-conjugant group *E*		2	3	1	5	1	3	1	3	13	65	73	7		
	Non-conjugants of 247*a*							1	1	3	8	9				
Dec. 23–27, 1930......	Ex-conjugant group *E*	2	11	4	1	1	2	2	7	5	23	35	58	15	1	
	Non-conjugants of 247*a*									4	9	8				

TABLE III

Comparison of non-conjugants of clone 247a with three sets (C, D, and E) of ex-conjugants derived from them at different times. Mean total numbers of fissions, with their standard deviations and coefficients of variation.

Experiment	Five-day periods	Group	Mean total number of fissions	Standard deviation	Coefficient of variation
1	Oct. 5–9, 1930	Ex-conjugant group *C*	10.65 ± 0.11	1.51 ± 0.07	14.21 ± 0.71%
		247*a* non-conjugants	10.18 ± 0.09	0.65 ± 0.07	6.38 ± 0.65%
	Oct. 10–14, 1930	Ex-conjugant group *C*	4.81 ± 0.11	1.58 ± 0.08	32.92 ± 1.83%
		247*a* non-conjugants	5.68 ± 0.22	1.45 ± 0.16	25.56 ± 2.97%
2	Oct. 24–28, 1930	Ex-conjugant group *D*	8.34 ± 0.23	2.89 ± 0.17	34.61 ± 2.20%
		247*a* non-conjugants	8.86 ± 0.37	2.07 ± 0.26	23.32 ± 3.13%
	Oct. 29–Nov. 2, 1930	Ex-conjugant group *D*	4.89 ± 0.17	1.79 ± 0.12	36.72 ± 2.74%
		247*a* non-conjugants	4.62 ± 0.16	0.84 ± 0.11	18.11 ± 2.47%
3	Dec. 18–22, 1930	Ex-conjugant group *E*	9.83 ± 0.11	2.11 ± 0.08	21.44 ± 0.80%
		247*a* non-conjugants	9.05 ± 0.15	1.07 ± 0.11	11.77 ± 1.21%
	Dec. 23–27, 1930	Ex-conjugant group *E*	9.13 ± 0.16	3.12 ± 0.12	34.12 ± 1.40%
		247*a* non-conjugants	9.19 ± 0.11	0.73 ± 0.08	7.96 ± 0.83%
	Ten-day periods				
1	Oct. 5–14, 1930	Ex-conjugant group *C*	15.70 ± 0.16	2.30 ± 0.12	14.68 ± 0.75%
		247*a* non-conjugants	15.78 ± 0.29	1.84 ± 0.21	11.68 ± 1.33%
2	Oct. 24–Nov. 2, 1930	Ex-conjugant group *D*	13.87 ± 0.27	2.89 ± 0.19	20.84 ± 1.44%
		247*a* non-conjugants	13.74 ± 0.21	1.05 ± 0.15	7.67 ± 1.11%
3	Dec. 18–27, 1930	Ex-conjugant group *E*	19.26 ± 0.23	4.37 ± 0.16	22.67 ± 0.88%
		247*a* non-conjugants	18.32 ± 0.17	1.08 ± 0.12	5.89 ± 0.76%

It is a remarkable fact that in spite of the great shifts of mode from period to period, the modes of the ex-conjugants of 247*a* and the modes of the non-conjugants of this clone were always very similar within any one period. Such a persistent similarity in modes appears in Table II. During the first experiment, the modes of ex-conjugants (using ex-conjugant group *C*) and non-conjugants are 11 and 10 for the first period, 5 and 6 for the second period; during the second experiment (using ex-conjugant group *D*) the modes are 10 and 9–10 for the first period, 4 and 4–5 for the second period; during the third experiment (using ex-conjugant group *E*) the modes are 11 and 10 for the first period, 11 and 9 for the second period. This close agreement of modal values of non-conjugant and ex-conjugant groups within the same period, in spite of great fluctuation in mode from period to period, must be due to some innate similarity between these two kinds of groups, both derived originally from the same ancestors.

TABLE IV

Ratio of ex-conjugant groups (C, D, and E) to non-conjugant groups of their parent clone (247a) in mean total number of fissions, standard deviations, and coefficients of variation. These ratios are calculated from the data in Table III.

Ten-day periods	Experiment	Ex-conjugant groups compared with groups of non-conjugants of 247*a*	Ratio of ex-conjugant group to synchronous group of non-conjugants of 247*a*		
			in mean total number of fissions	in standard deviation	in coefficient of variation
Oct. 5–14	1	C	1.0	1.3	1.3
Oct. 24–Nov. 2	2	D	1.0	2.8	2.7
Dec. 18–27	3	E	1.1	4.1	3.9

Tables III and IV demonstrate statistically the relations which Table II showed on inspection. In Table III the mean fission rate with its standard deviation and its coefficient of variation are given for both ex-conjugant and non-conjugant groups for all the periods of the experiments. In Table IV, the ratios of each of these statistical values for the ex-conjugant group to the corresponding values for the non-conjugant group are given. Again appears in Table III the great fluctuation in means from period to period. The ex-conjugant mean drops from 10.65 to 4.81 in the two periods of the first experiment and from 8.34 to 4.89 in the two periods of the second experiment. Also the means of the two groups in every period again appear remarkably similar: 10.65 and 10.18; 4.81 and 5.68; 8.34 and 8.86; 4.89 and 4.62; 9.83 and 9.05; 9.13 and 9.19. In three of the six periods the ex-conjugant mean exceeds the non-conjugant mean by an average of 0.51 fission; in the other three periods the ex-conjugant mean is less than the non-conjugant mean by an average of 0.48 fission. Thus the algebraic mean difference between the five-day means for the two groups is practically negligible. This similarity between the means of ex-conjugants and non-conjugants of the same race is expressed another way in Table IV where the ratio of the former to the latter is given. The ratios for the three experiments (total of ten days each) are 1.0, 1.0 and 1.1. The uniformity of this ratio of ex-conjugant mean to non-conjugant mean in all three experiments is the more remarkable because of the large differences in the absolute values of the means for the three experiments (Table III). In the first experiment the values are 15.70 and 15.78, in the second experiment 13.87 and 13.74; in the third experiment, 19.26 and 18.32. It is therefore quite clear that in the clone 247*a* the mean value of the fission rate is not changed by conjugation; nevertheless it should be remembered that some of the individual values, as well as the distribution of values, among the ex-conjugant groups are very different from those of the non-conjugant groups. So far as mean fission rate is concerned, there is in these experiments neither rejuvenescence nor depression as a result of conjugation.

For the purposes of the present paper, this is the most important result of these three experiments. But it should be remembered that so far as the separate lines are concerned, many ex-conjugants are very diverse from the non-conjugants. In the first experiment one ex-conjugant line reproduced more slowly than any non-conjugant line (13 others reproduced more slowly than all non-conjugants but one), 5 more rapidly, and 84 at the same rates as the non-conjugants; in the second experiment, 10 ex-conjugant lines reproduced more slowly than any non-conjugant line, 11 more rapidly, and 31 at the same rates as the non-conjugants; in the third experiment, 22 ex-conjugant lines reproduced more slowly than any non-conjugant lines, 95 more rapidly, and 50 at the same rates as the non-conjugants. Thus, in all three experiments, conjugation produced some rejuvenated lines, some depressed lines, and many lines that were unaltered in fission rate. It is only when all types of lines are averaged together that no effect of conjugation on fission rate appears. In this *average* absence of effect, all three experiments are in complete agreement.

The effect of conjugation on variability of fission rate has already been indicated. Table III shows that both the absolute variability, as measured by the standard deviation, and relative variability, as measured by the coefficient of variation, are greater for the ex-conjugants than for the non-conjugants, in every period of all three experiments. Conjugation has unmistakably increased the variation, both relative and absolute, of the fission rate. This result is in agreement with the results of Jennings (1913), Raffel (1930), and Jennings *et al.* (1932). In Table IV are given the ratios of the ex-conjugant standard deviations and coefficients of variation to the corresponding values for the non-conjugants. Because of the close agreement of the means of corresponding groups of ex-conjugants and non-conjugants, little difference appears between the ratios of the standard deviations and the ratios of the coefficients of variation in any one experiment. The two ratios differ very much, however, from experiment to experiment. For the first experiment both ratios are 1.3; for the second experiment, the ratios are 2.8 and 2.7, respectively; for the third experiment, 4.1 and 3.9, respectively. Thus conjugation has increased both relative and absolute variation in fission rate in all three experiments. In each case it has given rise to lines with higher fission rate, and lines with lower fission rate, as well as to lines with unchanged fission rate.

As to the relative mortality of conjugants and non-conjugants, no constant result was found in these three experiments. In the first experiment the mortality was 22.4 per cent for the ex-conjugant lines, 20.8 per cent for the non-conjugant lines; in the second experiment, 44.1 per cent for the ex-conjugants, 31.2 per cent for non-conjugants; in the third experiment, 10.2 per cent for ex-conjugants, 20.8 per cent for non-conjugants. Thus the mortality is nearly alike in the two groups in the first experiment, it is greater among the ex-conjugants in the second experiment, and is greater among the non-conjugants in the third experiment.

The three experiments on the effects of conjugation in the race 247*a* have thus shown, as far as they go, that conjugation does not always produce the same effect on mortality in this race, but that it does always produce the same effect on mean fission rate, on absolute variability, and on relative variability. The mean fission rate is regularly unchanged by conjugation, so that conjugation neither rejuvenates nor depresses the population as a whole. However, both absolute variability and relative variability of fission rate are regularly increased by conjugation. This results, in spite of absence of the effect on the mean, in the regular production of both depressed and rejuvenated ex-conjugants.

IV. Simultaneous Conjugations within Six Ex-conjugant Clones Derived from the Clone 247*a*

The preceding section brought out the fact that in clone 247*a*, although the *relations* of ex-conjugants to non-conjugants remained the same in different periods, the *absolute values* for both ex-conjugants and non-conjugants were very different in different periods. This showed the desirability of conducting experimental comparisons between different clones cultivated in the same medium at the *same time*. Therefore, in an attempt to discover whether conjugation produces different effects in different clones, we compared the effects of *simultaneous* conjugations in *six* clones. To make the issue sharper, we deliberately selected six closely related clones that had similar characteristics. These were six of the most rapidly reproducing sister ex-conjugant clones (*E40a, E41a, E46b, E80b, E81a* and *E85b*) produced in the *E* conjugation of the clone 247*a* (see Fig. 1). The conjugations induced in them on January 12–14 (see Table I) were designated the *F, G, L, K, J,* and *H* conjugations, respectively. Records of fission rates and mortality among the conjugants and corresponding non-conjugants were kept until January 25.

From these data an answer to the question of the possible difference in effect of conjugation in different clones may be sought in two ways. On the one hand, the groups of ex-conjugants of the six clones may be compared as to fission rate, variability and mortality. On the other hand, the ex-conjugants of each clone may be compared with the non-conjugants of the same clone. For the latter comparison the data are not so extensive as could be desired, since it was impossible, for technical reasons, to keep in progress a large number of non-conjugant lines at the same time with the numerous ex-conjugant lines. But experience has shown that a small sample of non-conjugants usually gives a fair picture of the general character of the non-conjugant population, there being, as a rule, little variation among the members of a single clone multiplying vegetatively. On this account we feel that the comparison of the ex-conjugants with the small group of 6 or 8 lines of non-conjugants from the same clone is of interest, though of less value than would be the case if the non-conjugant groups were larger.

We take up first the comparison of the groups of ex-conjugants from the six clones. Table V gives the distribution of total number of fissions in two five-day periods (January 16–20 and 21–25) among the ex-conjugants from each clone. From these data may be calculated means, standard deviations and coefficients of variation of fission rate; these are given in Table VI. Among the six groups of ex-conjugants there are three (*E41a, E80b,* and *E81a*) which have consistently different mean fission rates in both five-day periods. *E41a* is consistently highest, *E80b* consistently intermediate, and *E81a* consistently lowest. Their mean fission rates are 16.69, 16.38, and 7.46 for the first period, 13.66, 13.26 and 5.39 for the second period. The differences between

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TABLE V

Comparison of groups of non-conjugants of six clones (E40a, E41a, E46b, E80b, E81a, E85b) with the ex-conjugant groups derived from them. Distribution of total numbers of fissions in two five-day periods.

Clone	Period	Group	Total number of fissions																				
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E40a	Jan. 16–20....	Ex-conjugants											1				9	13	24	21	18	1	
		Non-conjugants														3	1	3	1				
	Jan. 21–25....	Ex-conjugants								1	1	1	1	1	7	18	27	22	5				
		Non-conjugants											1		1	3	1	2					
E41a	Jan. 16–20....	Ex-conjugants						1	1	1		1			1	1	3	7	8	23	25	13	1
		Non-conjugants																1	2	3	2		
	Jan. 21–25....	Ex-conjugants			1	1			1		3		2	1	1	12	23	35	5				
		Non-conjugants												1	1	1	3	2					
E46b	Jan. 16–20....	Ex-conjugants					1	1		1		1		2		2	4	6	13	22	18	7	1
		Non-conjugants															1		1	3	1		
	Jan. 21–25....	Ex-conjugants						2			1		1	4	3	8	24	21	6				
		Non-conjugants										1		1	2	1	1	1					
E80b	Jan. 16–20....	Ex-conjugants							1			3		2				5	19	23	17	6	
		Non-conjugants												1			1	3	2				
	Jan. 21–25....	Ex-conjugants			2	1	1		1			1	1	3	1	5	32	23	1				
		Non-conjugants												1	2	1		2					
E81a	Jan. 16–20....	Ex-conjugants	2	1	4		2	2	6	3	6	3	3	2	2	2	1	1	1				
		Non-conjugants												1	1	1	2		2				
	Jan. 21–25....	Ex-conjugants	1	3	1		3	2	2	1	1	1	2				1						
		Non-conjugants						2		1		1				1	1						
E85b	Jan. 16–20....	Ex-conjugants	1	2					2		1	1	1		2		3	6	11	28	9	4	
		Non-conjugants															1	1	3	1			
	Jan. 21–25....	Ex-conjugants						1	1					3	2	10	22	18	3				
		Non-conjugants											1			1	2	3					

TABLE VI

Comparison of groups of non-conjugants of six clones (E40a, E41a, E46b, E80b, E81a, E85b) with groups of ex-conjugants derived from them. Mean total numbers of fissions, with their standard deviations and coefficients of variation.

Period	Clone	Mean total number of fissions		Standard deviations		Coefficients of variation	
		Ex-conj.	Non-conj.	Ex-conj.	Non-conj.	Ex-conj.	Non-conj.
Jan. 16–20, 1931	*E40a*	16.26 ± 0.10	14.25 ± 0.26	1.44 ± 0.07	1.09 ± 0.18	8.87 ± 0.46%	7.65 ± 1.30%
	E41a	16.69 ± 0.19	16.75 ± 0.23	2.66 ± 0.14	0.97 ± 0.16	15.97 ± 0.84%	5.78 ± 0.98%
	E46b	16.18 ± 0.22	16.50 ± 0.35	2.88 ± 0.16	1.26 ± 0.25	17.81 ± 0.99%	7.63 ± 1.49%
	E80b	16.38 ± 0.19	14.57 ± 0.41	2.40 ± 0.13	1.59 ± 0.29	14.62 ± 0.82%	10.92 ± 1.99%
	E81a	7.46 ± 0.42	13.71 ± 0.45	3.98 ± 0.30	1.75 ± 0.32	53.38 ± 4.98%	12.76 ± 2.34%
	E85b	15.31 ± 0.33	15.67 ± 0.26	4.06 ± 0.23	0.94 ± 0.18	26.53 ± 1.60%	6.02 ± 1.18%
Jan. 21–25, 1931	*E40a*	13.70 ± 0.12	13.13 ± 0.37	1.60 ± 0.08	1.54 ± 0.26	11.69 ± 0.62%	11.70 ± 2.00%
	E41a	13.66 ± 0.18	13.50 ± 0.32	2.52 ± 0.14	1.32 ± 0.22	18.44 ± 1.09%	9.80 ± 1.67%
	E46b	13.70 ± 0.17	12.29 ± 0.47	2.10 ± 0.12	1.83 ± 0.33	15.33 ± 0.89%	14.89 ± 1.98%
	E80b	13.26 ± 0.24	13.00 ± 0.42	2.98 ± 0.17	1.53 ± 0.30	22.44 ± 1.32%	11.75 ± 2.32%
	E81a	5.39 ± 0.59	8.83 ± 0.99	3.68 ± 0.41	3.58 ± 0.70	68.36 ± 10.69%	40.51 ± 9.09%
	E85b	13.73 ± 0.17	13.71 ± 0.43	1.90 ± 0.12	1.67 ± 0.30	13.81 ± 0.87%	12.15 ± 2.22%

*E*41*a* and *E*81*a* are 9.23 ± 0.45 for the first period and 8.27 ± 0.62 for the second period. The differences between *E*80*b* and *E*81*a* are 8.92 ± 0.45 for the first period and 7.87 ± 0.64 for the second period. The differences between *E*41*a* and *E*80*b* are 0.31 ± 0.27 for the first period and 0.40 ± 0.30 for the second period. Thus the differences between *E*41*a* and *E*81*a*, and the differences between *E*80*b* and *E*81*a* are clearly significant, the differences ranging from twelve to twenty times their probable errors. On the other hand, the differences between *E*41*a* and *E*80*b* are only 1.1 to 1.3 greater than their probable errors and therefore cannot be considered as certainly significant. The uniformity of these differences between *E*41*a* and *E*80*b*, however, makes them somewhat more significant than their probable errors would indicate. It is clear, in any case, that there is a well-defined difference in fission rate between *E*81*a* and all the other groups. The differences between *E*81*a* and the group nearest it in each period are 7.85 ± 0.53 (*E*85*b*–*E*81*a*) in the first period and 7.87 ± 0.64 (*E*80*b*–*E*81*a*) in the second period.

TABLE VII

Differences in standard deviation among the three grades of ex-conjugant groups. Grade 1 = group *E*40*a;* grade 2 = groups *E*41*a*, *E*46*b*, and *E*80*b;* grade 3 = group *E*81*a*.

Grades Compared	Groups Compared	Period 1		Period 2	
		Difference	Difference / P.E.	Difference	Difference / P.E.
3–2....	*E*81*a*–*E*46*b*	1.10 ± 0.34	3.2	1.58 ± 0.43	3.7
3–2....	*E*81*a*–*E*41*a*	1.32 ± 0.33	4.0	1.16 ± 0.43	2.7
3–2....	*E*81*a*–*E*80*b*	1.58 ± 0.33	4.8	0.70 ± 0.44	1.6
2–1....	*E*41*a*–*E*40*a*	1.22 ± 0.16	7.6	0.92 ± 0.16	5.8
2–1....	*E*46*b*–*E*40*a*	1.44 ± 0.18	8.0	0.50 ± 0.14	3.6
2–1....	*E*80*b*–*E*40*a*	0.96 ± 0.14	6.9	1.38 ± 0.19	7.3
3–1....	*E*81*a*–*E*40*a*	2.54 ± 0.31	8.2	2.08 ± 0.42	5.0

In absolute variability, Table VI shows that the six ex-conjugant groups fall into three consistently distinct grades: one with standard deviation near 1.5 fissions, one between 2.0 and 3.0 fissions, and one between 3.7 and 4.0 fissions, for the two five-day periods. The group *E*40*a* had standard deviations of 1.44 and 1.60 fissions; these were lower than those of any other group. The groups *E*41*a*, *E*46*b*, and *E*80*b* all fell in an intermediate grade with standard deviations varying from 2.0 to 3.0 fissions and without consistent differences among the three groups in this grade. The group *E*81*a*, with standard deviations of 3.98 and 3.68 fissions had a variation distinctly higher than any other group.

Not only are the differences between these three grades of standard deviations consistent, but they are also statistically significant, as appears in Table VII. All the differences except those in the second period between *E*41*a* and *E*81*a* and between *E*80*b* and *E*81*a* are more than three times their probable error. Therefore the differences between the three groups *E*40*a*, *E*46*b*, and *E*81*a* are clearly significant and establish the existence of three grades of standard deviations among the six races.

The data on relative variability of fission rate, as measured by the coefficient of variation are also given in Table VI. Differences in relative variability among the ex-conjugant groups closely parallel the differences in absolute variability. The group *E*40*a* is again the least variable with coefficients of 8.87 per cent and 11.69 per cent; *E*41*a*, *E*46*b*, and *E*80*b* again fall into the intermediate grade, with coefficients from 14.62 per cent to 22.44 per cent; and *E*81*a* is again the most variable, with coefficients of 53.38 per cent and 68.36 per cent. As appears in Table VIII, the differences between these three grades are clearly statistically significant in every case and in both periods.

TABLE VIII

Differences in coefficients of variation among the three grades of ex-conjugant groups. Grade 1 = group *E*40*a*; grade 2 = groups *E*41*a*, *E*46*b*, and *E*80*b*; grade 3 = group *E*81*a*.

Grades Compared	Groups Compared	Period 1		Period 2	
		Difference	Difference / P.E.	Difference	Difference / P.E.
3–2....	*E*81*a*–*E*80*b*	38.76 ± 5.05	7.7	45.92 ± 10.77	4.3
3–2....	*E*81*a*–*E*46*b*	35.57 ± 5.08	7.0	53.03 ± 10.72	5.0
3–2....	*E*81*a*–*E*41*a*	37.41 ± 5.05	7.4	49.92 ± 10.74	4.7
2–1....	*E*80*b*–*E*40*a*	5.75 ± 0.94	6.1	10.75 ± 1.46	7.4
2–1....	*E*46*b*–*E*40*a*	8.94 ± 1.01	8.9	3.64 ± 1.08	3.4
2–1....	*E*41*a*–*E*40*a*	7.10 ± 0.96	7.4	6.75 ± 1.21	5.6
3–1....	*E*81*a*–*E*40*a*	44.51 ± 5.00	8.9	56.67 ± 10.71	5.3

The data on mortality, as measured by the ratio of the number of lines that died out during a given period to the number of lines that were alive at the beginning of the period can be expressed as percentages. These percentages, in the cases of the groups of ex-conjugants, are the percentages of lines, or ex-conjugant clones (since only one line of each ex-conjugant clone was in progress), that died out during a given period. The percentages of mortality among the six groups of ex-conjugant clones, during the whole experiment of January 16–25, fell into three distinctly different grades.

The groups *E*40*a* and *E*41*a* had a very low mortality (12.5 per cent and 11.5 per cent, respectively); the groups *E*46*b*, *E*80*b* and *E*85*b* had a moderate mortality (35.7 per cent, 24.2 per cent, and 37.5 per cent, respectively); and the group *E*81*a* had a very high mortality (81.3 per cent). In these differences there appears some foundation for the conflicting results of different investigators concerning the effect of conjugation on mortality. *E*40*a* and *E*41*a* are clones of such genetic constitution that conjugation results in but a small proportion of non-viable combinations of nuclear material; on the other hand, *E*81*a* is a clone whose genetic constitution is such that, under the same conditions of experiment, a very high proportion of non-viable combinations result from conjugation. The question of the effect of conjugation on mortality cannot properly be asked in the form "What is the effect of conjugation on mortality?" for the answer will differ in different cases; the answer which will be discovered in any particular case depends upon the genetic constitution of the race under examination. In some races conjugation will result in very great mortality; in other races, in very little mortality.

In the preceding account of differences among the groups of ex-conjugants derived from six related clones, the most outstanding features were the great differences between ex-conjugants from the clone *E*81*a* and ex-conjugants from all the other clones. These differed in mean fission rate, in relative variability, in absolute variability, and in mortality. In dealing with Protozoa studied in daily isolation culture, the possibility must always be kept in mind that differences observed between the groups investigated might result from a diversity in the bacterial flora of the culture fluid such as might be perpetuated from day to day. Although we were always aware of this danger and, as a routine part of our experimental procedure, took numerous precautions to avoid it, we thought it best to make a direct test in the case of the clone *E*81*a*. The test consisted in retaining the twenty-one separate drops of the culture fluid in which twenty-one ex-conjugant lines from *E*81*a* had died and transferring to each of these drops a single specimen from one of the normal lines of one of the other groups of ex-conjugants. To each of these drops, one extra drop of fresh fluid was added, and the paramecia left overnight in the combination of old fluid and fresh fluid. Next day and thereafter, each line was treated in the usual manner, being transferred daily to a drop of fresh culture fluid. As controls, we kept a sister animal of each of the paramecia put into the death-fluid of the *E*81*a* lines; these were cultivated in the usual culture medium, not mixed with the fluid in which the *E*81*a* ex-conjugants had died. The records of reproductive activity during the following five days for the test animals and controls were practically identical. It is certain, therefore, that the differences between the groups of ex-conjugants of *E*81*a* and the other ex-conjugant groups cannot be attributed to self-perpetuating differences in the conditions of cultivation. The only alternative is that the differences found are truly constitutional.

The phenomena observed among the non-conjugants of *E*81*a* give further evidence that this clone differed constitutionally from the others. The original *E*81*a* clone, as well as the other five clones, were selected for study because preliminary observations had shown them to be the most rapid reproducers that we had in the laboratory. The astonishing inviability and low fission rates of the *E*81*a* ex-conjugants was the first evidence that this race differed markedly from the others. Later, however, the non-conjugants themselves underwent a sudden and marked change of character. The mean fission rate dropped from about three divisions a day to less than two divisions a day. This abrupt change occurred in the non-conjugants about one week after other members of the clone had been allowed to conjugate. The difference between the ex-conjugants of *E*81*a* and the ex-conjugants of the other clones was apparent immediately after conjugation. The change thus appeared one week earlier in the ex-conjugants than in the non-conjugants. This, it seems, is further strong evidence that the clone *E*81*a* was constitutionally different from the other clones: such difference, brought to light first in ex-conjugants from this clone, later appeared in the non-conjugants themselves.

Comparison of the non-conjugants and the ex-conjugants of other clones also brings out a relation of much interest. The diversities in the fission rate among the three ex-conjugant groups *E*41*a, E*80*b,* and *E*81*a* (see p. 271 above), are paralleled by the diversities in fission rate among their three non-conjugant groups (see Table VI). For the first period, the mean number of fissions attained by *E*41*a* non-conjugants was 16.75; by *E*80*b* non-conjugants 14.57; by *E*81*a* non-conjugants 13.71. For the second period, the non-conjugant mean attained by *E*41*a* was 13.50; by *E*80*b,* 13.00; by *E*81*a,* 8.83. It is remarkable that it is not possible to select any two clones from among the six studied, in which the clone having the faster multiplying non-conjugants consistently has the slower multiplying ex-conjugants. On the other hand, it is possible to select six pairs of clones (each of the five others compared with *E*81*a*, and *E*41*a* compared with *E*80*b*) in which both the non-conjugants and the ex-conjugants of the one consistently multiply more rapidly than the corresponding groups of the other. All of the parent non-conjugant clones except *E*81*a* were so similar in mean fission rate that it is surprising that even one pair of clones (*E*41*a* and *E*80*b*) could be selected with consistent differences from period to period. These results are similar to the results of Calkins (1919 and 1920), Woodruff and Spencer (1924), and Jennings *et al.* (1932), in that diversities in mean fission rate between different lines of descent within the same species are found to persist through conjugation, reappearing as similar diversities between the derived sets of ex-conjugants.

TABLE IX

Comparison of six clones in the ratios of ex-conjugants to non-conjugants in mean total number of fissions, standard deviation, and coefficient of variation

	Period	Clones					
		*E*40*a*	*E*41*a*	*E*46*b*	*E*80*b*	*E*81*a*	*E*85*b*
Mean total number of fissions . .	Jan. 16–20	1.14	1.00	0.98	1.12	0.54	0.98
	Jan. 21–25	1.04	1.01	1.12	1.02	0.61	1.00
Standard deviation	Jan. 16–20	1.32	2.74	2.29	1.51	2.27	4.32
	Jan. 21–25	1.04	1.91	1.15	1.95	1.03	1.14
Coefficient of variation	Jan. 16–20	1.16	2.76	2.33	1.34	4.18	4.41
	Jan. 21–25	1.00	1.88	1.03	1.91	1.69	1.14

In Table IX are given the ratios of ex-conjugant mean fission rates, standard deviations, and coefficients of variation to the corresponding values for non-conjugants. The ratios of the mean fission rates fall into three classes: those less than one, those equal to one, and those greater than one. The ratios for the clone *E*81*a* are 0.54 for the first period, and 0.61 for the second period. The corresponding ratios for the clone *E*41*a* are 1.00 and 1.01; and for the clone *E*80*b* they are 1.12 and 1.02. Thus, the mean fission rate of the clone *E*81*a* has been markedly decreased by conjugation; the mean fission rate of the clone *E*41*a* has been unchanged by conjugation; and the mean fission rate of the clone *E*80*b* has been very slightly increased by conjugation. With respect to the effect of conjugation on mean fission rate, *E*41*a* is a clone similar to 247*a*, as described above on page 268. This simultaneous comparison of the effects of conjugation in six clones demonstrates that conjugation does not have the same effect on mean fission rate in all clones: in some clones, conjugation raised very slightly the mean fission rate (rejuvenescence); in other clones, it lowered the mean fission rate (depression); and in other clones it left the mean fission rate unaltered. It appears here again that *the effect of conjugation on mean fission rate depends on the nature of the clone which conjugates.*

The ratios of the standard deviations in the ex-conjugants to those in the non-conjugants (Table IX) show a number of consistent differences, but most of them are slight. In *E*40*a* the ratio is lower than in *E*85*b*, *E*41*a*, *E*46*b*, *E*80*b*; in *E*81*a* it is lower than in *E*85*b* and *E*41*a*; and in *E*46*b* it is lower than in *E*41*a*. The greatest consistent difference in ratio is between *E*40*a* and *E*41*a*. The ratios for the former are 1.32 and 1.04, for the two different periods; for the latter the corresponding ratios are 2.74 and 1.91. Thus conjugation increased absolute variability of fission rate about twice as much in the clone *E*41*a* as in the clone *E*40*a*.

TABLE X

Comparison of clone E40a with clone E41a in differences between non-conjugants and ex-conjugants in standard deviation and coefficient of variation of total number of fissions.

		Jan. 16–20	Jan. 21–25
Difference between non-conjugants and ex-conjugants in Standard Deviations	Clone *E*40*a*	0.33 ± 0.19	0.06 ± 0.27
	Clone *E*41*a*	1.69 ± 0.21	1.20 ± 0.26
Difference between non-conjugants and ex-conjugants in Coefficients of Variation	Clone *E*40*a*	1.22 ± 1.38%	0.01 ± 2.09%
	Clone *E*81*a*	10.19 ± 1.18%	8.64 ± 1.99%

The ratios of the coefficients of variation of the ex-conjugants to those of the non-conjugants (also in Table IX) show differences similar to the differences in the ratios of the standard deviations. *E*40*a* is consistently lowest, with ratios of 1.16 and 1.00; *E*41*a* is among the highest, with ratios of 2.76 and 1.88. Thus the relative variability of *E*41*a* has been about twice as much increased by conjugation as the relative variability of *E*40*a*.

These results are another instance of the diversity of the effects of conjugation in different clones. In the clone *E*40*a*, conjugation has not significantly increased either absolute or relative variability. On the other hand, in the clone *E*41*a*, conjugation has increased both absolute and relative variability by 100 per cent to 200 per cent.

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TABLE XI

Comparison of non-conjugants of clone E40a with non-conjugants of clone E81a, and of groups of ex-conjugants of clone E40a with groups of ex-conjugants of clone E81a, in four different conjugations. Distribution of total number of fissions in five-day periods.

Experiment	Period	Clones	Total number of fissions in five-day periods in ex-conjugant groups																			
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Jan. 16–20	*E40a*											1				9	13	24	21	18	1
		E81a	2	1	4		2	2	6	3	6	3	3	2	2	2	1	1	1			
	Jan. 21–25	*E40a*								1	1	1	1	1	7	18	27	22	5			
		E81a	1	3	1		3	2	2	1	1	1	2				1					
2	Feb. 12–14	*E40a*	4	7	1	3	8	12	11	22	11	5										
		E81a	4	8	3	3	4	5	1	1	1											
3	Feb. 19–23	*E40a*				1	2	4		2	3	5	4	5	7	6	7					
		E81a	1	2	4	4	5	10	3	7	6	3		3	1	1	1					
	Feb. 24–28	*E40a*		1	1	4		1	1		2	2	9	3		3						
		E81a				1																
4	Mar. 18–22	*E40a*	1		1				1		1	1	5	3	11	2						
		E81a		1		1	2			1			1									
	Mar. 23–27	*E40a*						1		3		2	1	1	7	7						
		E81a						2						1								
	Mar. 28–Apr. 1	*E40a*						1				1		4	1	5	4	4	1			
		E81a							1		1						1					

TABLE XI—*Continued*

Experiment	Period	Clones	Total number of fissions in five-day periods in non-conjugant groups																			
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Jan. 16–20	*E40a*														3	1	3	1			
		E81a												1	1	1	2		2			
	Jan. 21–25	*E40a*											1		1	3	1	2				
		E81a						2		1		1				1	1					
2	Feb. 12–14	*E40a*									1	4	3									
		E81a								1	5	10										
3	Feb. 19–23	*E40a*										1	1	2	1		2					
		E81a											2	1	1	2	4					
	Feb. 24–28	*E40a*										1	1									
		E81a				1	1	1				1										
4	Mar. 18–22	*E40a*	1						1				2	1	1	1	1					
		E81a		1	1	2	2		4	2	1											
	Mar. 23–27	*E40a*						1	3			1	1	1	1							
		E81a	1	1	2	3	3	2		1	1											
	Mar. 28–Apr. 1	*E40a*													1	4	4	1				
		E81a							3		2	3	1	1								

As appears in Table X, the diversities between ex-conjugants and non-conjugants in *E*40*a* are small and insignificant, but in *E*41*a* they are large and clear. Thus an experiment on *E*40*a* alone would have led to the conclusion that conjugation does not increase variation; but an experiment on *E*41*a* alone would have led to the conclusion that conjugation greatly increases variability. The present experiment shows that neither conclusion tells the whole truth. The effect of conjugation on variability, like its effect on mean fission rate, depends upon the nature of the race which conjugates. In either characteristic, some races will manifest one effect of conjugation, other races a different or even opposite effect.

Comparison of the percentage mortality of the ex-conjugants with the percentage mortality of the non-conjugants brings out the same types of relations. The non-conjugants of clone *E*85*b* had a mortality of 37.5 per cent which was exactly the same as the percentage mortality among the group of ex-conjugants from this clone. In clone *E*80*b*, the non-conjugants had a mortality of 25.0 per cent; the ex-conjugants, 24.2 per cent. Clearly conjugation produced practically no change in the mortality of clones *E*80*b* and *E*85*b*. On the other hand, the non-conjugants of clone *E*81*a*, like the non-conjugants of clone *E*85*b*, had a mortality of 37.5 per cent, but the ex-conjugants of clone *E*81*a* had a mortality of 81.3 per cent. Thus, as with mean fission rate and variability, in some clones (*e.g.*, *E*81*a*), conjugation greatly alters mortality, and in other clones leaves mortality unchanged.

The evidence thus far shows clearly that diversities in the effect of conjugation in different clones are due to constitutional diversities between the clones. It seems desirable to discover whether such diverse effects in different clones are constant. Will the same diversities recur in repeated conjugations? This matter was tested in two of the clones that were found to give diverse results of conjugation; it is fully discussed in the next section.

V. REPEATED COMPARISONS OF THE EFFECTS OF CONJUGATIONS IN THE TWO CLONES *E*40*a* AND *E*81*a*

Conjugations within *the Clones E40a and E81a*

In order to determine whether the diverse effects of conjugation in different clones occur regularly, four sets of conjugants were obtained and studied in clones *E*40*a* and *E*81*a*. These eight sets of conjugants were obtained two at a time, one from each of the two clones. In every case, of course, conjugation in the two clones occurred in lines that had not conjugated since the *E* conjugation.

Results of the four sets of conjugations were studied during the intervals: January 16–25, February 12–14, February 19–28, and March 18–April 1. The experiments, as usual, were designed to compare mortality and fission rates, and variability of fission rates. As will appear in detail below, mortality among the ex-conjugants from the clone *E*81*a* was so high that, although comparison of rates of mortality could be made for each of the four experiments, comparisons of fission rate, variability of fission rate, and the ratios of the data of ex-conjugants to the data of non-conjugants in these respects could not be made for four of the eight periods of the experiments. During the remaining four periods (January 16–20, January 21–25, February 12–14, February 19–23), however, there were 41, 18, 30, and 51 ex-conjugant lines of *E*81*a* to compare with 87, 84, 84, and 46 ex-conjugant lines of *E*40*a*, so that statistical comparisons of fission rate and variability could be made.

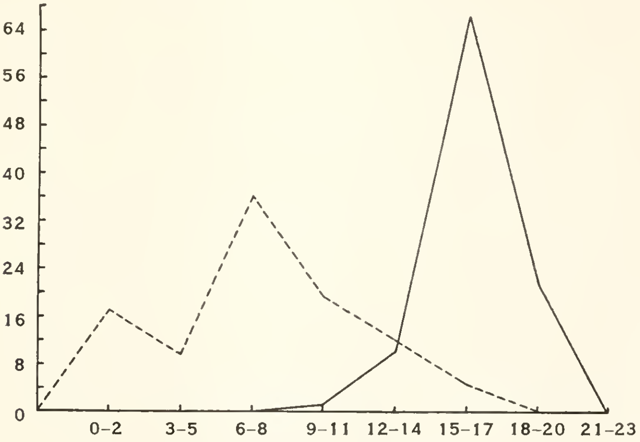

FIG. 2. Comparison of group of ex-conjugants from clone *E*40*a* (solid curve) with a group of ex-conjugants from *E*81*a* (broken curve), during the five-day period, January 16–20. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

The basic data for all eight periods of these four experiments are given in Tables XI and XIII. Table XI gives the basic data on fission rates, from which all the data on means, standard deviations and coefficients of variation are derived. Table XIII gives the data on mortality.

The most striking fact shown in Table XI is the difference between the distribution of values for the ex-conjugants of *E*81*a* and the distribution of values for the ex-conjugants of *E*40*a*. The former are obviously massed considerably to the left of (that is, are lower in value than) the latter. This difference in distribution is brought out graphically by Figs 2, 3, 4, and 5; these are graphs on which are plotted the percentage of ex-conjugants attaining each number of fissions during a single five-day period (three-day period in Fig. 4). Figures 2 and 3 give the curves for the two periods of the first experiment; Fig. 4 gives the curves for the short second experiment; Fig. 5, the curves for the first period of the third experiment. In all periods of the fourth experiment too few ex-conjugants of *E*81*a* survived to warrant plotting curves.

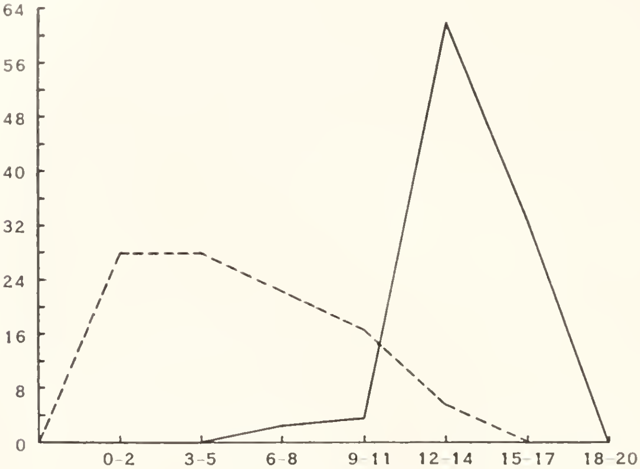

FIG. 3. Comparison of group of ex-conjugants from clone *E*40*a* (solid curve) with a group of ex-conjugants from *E*81*a* (broken curve), during the five-day period, January 21–25. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

In the first experiment (Figs. 2 and 3), the curves for the two groups of ex-conjugants are totally different. The solid lines, representing the groups of ex-conjugants from *E*40*a*, are curves peaked sharply between 15 and 17 fissions in the first period (Fig. 2) and between 12 and 14 fissions in the second period (Fig. 3). On the other hand, the broken lines, representing the groups of ex-conjugants from *E*81*a*, are long, low, and irregular, extending from 0 to 17 in the first period (Fig. 2), and from 0 to 14 in the second period (Fig. 3). The curves thus bring out the much greater range and variation, but much greater frequency of lower fission rates in the *E*81*a* group as compared with the *E*40*a* group.

In both the second experiment and the first period of the third experiment (Figs. 4 and 5), the curves for the two groups have nearly the same range, but the curves for *E*40*a* are peaked near their upper limits and the curves of *E*81*a* near their lower limits. In the second experiment (Fig. 4) the peak of the *E*40*a* curve is between 6 and 8; the peak of the *E*81*a* curve between 0 and 2. In the first period of the third experiment (Fig. 5), the peak of the *E*40*a* curve is at 12–14 fissions; the peak of the *E*81*a* curve at 3–5 fissions. These curves bring out the much more frequent occurrence of low fission rates among the *E*81*a* ex-conjugants as compared with the *E*40*a* ex-conjugants; and also the very similar range and absolute variation. The relative variability, nevertheless, is much greater among the *E*81*a* groups of ex-conjugants on account of their much lower means. Both of these sets of curves (Figs. 4 and 5) agree with the former sets (Figs. 2 and 3) in that the main portions of the curves for the *E*40*a* groups lie to the right of the main

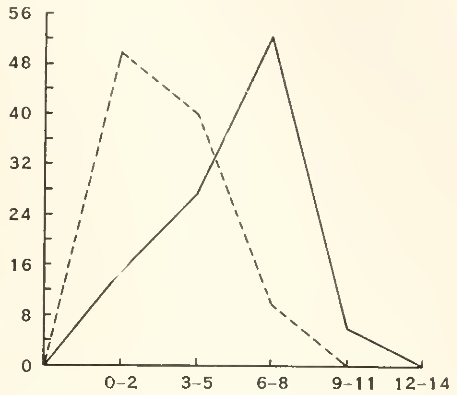

FIG. 4. Comparison of group of ex-conjugants from clone *E*40*a* (solid curve) with a group of ex-conjugants from *E*81*a* (broken curve), during the three-day period, February 12–14. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

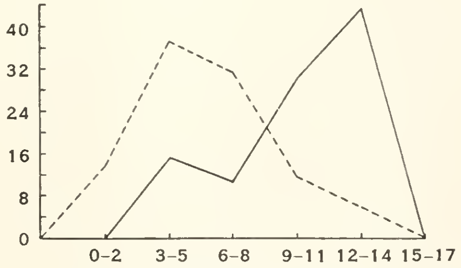

FIG. 5. Comparison of group of ex-conjugants from clone *E*40*a* (solid curve) with a group of ex-conjugants from *E*81*a* (broken curve), during the five-day period, February 19–23. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

portions of the corresponding *E*81*a* curves; that is, the majority of ex-conjugants from clone *E*40*a* have higher fission rates than the majority of ex-conjugants from clone *E*81*a*, during corresponding periods.

Table XII gives for all groups the mean fission rates, their standard deviations, and their coefficients of variation for all periods yielding statistically significant data. In every period the mean fission rate of the ex-conjugants of *E*40*a* is significantly higher than the mean fission rate of the ex-conjugants of *E*81*a*: 16.26 as compared with 7.46; 13.70 with 5.39; 5.51 with 2.83; 10.41 with 6.00. Thus the statistics on mean fission rate agree with the relation brought out graphically in Figs. 2, 3, 4, and 5; namely, that conjugation in the clone *E*40*a* uniformly yields groups of ex-conjugants with higher mean fission rates than does conjugation in the clone *E*81*a*.

The standard deviations do not show consistent significant differences between the two groups of ex-conjugants. In the first experiment, the *E*81*a* group has significantly greater standard deviations than the *E*40*a* group: 3.98 as compared with 1.44; and 3.68 as compared with 1.60. But in the second and third experiments the differences are not significant: 2.19 as compared with 2.12, and 3.13 as compared with 3.18. Again, the statistics show, in another way, the fact brought out in Figs. 2, 3, 4, and 5; namely, that the range in fission rate of biotypes produced by conjugation is not consistently different in the two groups.

The coefficients of variation, however, do show consistently significant differences. The coefficients of variation of the *E*81*a* groups of ex-conjugants are always greater than the coefficients of variation of the *E*40*a* groups of ex-conjugants: 53.38 per cent as compared with 8.87 per cent; 68.36 per cent as compared with 11.69 per cent; 77.38 per cent with 38.43 per cent; 52.08 per cent with 30.52 per cent. This regularly higher coefficient of variation in groups of ex-conjugants of clone *E*81*a*, together with the lack of consistent difference in standard deviations, can be due to but one thing: the lower mean fission rate of the *E*81*a* groups. This situation with respect to relative variability (coefficient of variation) and its dependence on differences in the means was indicated, it will be recalled, by Figs. 2, 3, 4, and 5, as mentioned above.

Table XIII gives the percentages of mortality in the different groups. In each experiment the mortality of the *E*81*a* group of ex-conjugants is very much greater than the mortality of the *E*40*a* group of ex-conjugants: 81.3 per cent as compared with 12.5 per cent; 40.9 per cent with 14.3 per cent; 99.5 per cent with 71.9 per cent; 92.9 per cent with 47.5 per cent. For the four experiments the average mortality among the groups of ex-conjugants from clone *E*81*a* was 81.3

TABLE XII

Comparison of groups of non-conjugants of clones E40a and E81a and of groups of ex-conjugants derived from each. Mean total number of fissions, with their standard deviations and coefficients of variation.

	Exp.	Period	Ex-conjugants		Non-conjugants	
			E40a	*E81a*	*E40a*	*E81a*
Mean total number of fissions..........	1	Jan. 16–20	16.26 ± 0.10	7.46 ± 0.42	14.25 ± 0.26	13.71 ± 0.45
		Jan. 21–25	13.70 ± 0.12	5.39 ± 0.59	13.13 ± 0.37	8.83 ± 0.99
	2	Feb. 12–14	5.51 ± 0.16	2.83 ± 0.27	9.25 ± 0.16	8.56 ± 0.10
	3	Feb. 19–23	10.41 ± 0.32	6.00 ± 0.30	11.57 ± 0.45	12.50 ± 0.34
Standard deviation..................	1	Jan. 16–20	1.44 ± 0.07	3.98 ± 0.30	1.09 ± 0.18	1.75 ± 0.32
		Jan. 21–25	1.60 ± 0.08	3.68 ± 0.41	1.54 ± 0.26	3.58 ± 0.70
	2	Feb. 12–14	2.12 ± 0.11	2.19 ± 0.19	0.66 ± 0.11	0.61 ± 0.07
	3	Feb. 19–23	3.18 ± 0.22	3.13 ± 0.21	1.76 ± 0.32	1.57 ± 0.24
Coefficient of variation...............	1	Jan. 16–20	8.87 ± 0.46%	53.38 ± 4.98%	7.65 ± 1.30%	12.76 ± 2.34%
		Jan. 21–25	11.69 ± 0.62%	68.36 ± 10.69%	11.70 ± 2.00%	40.51 ± 9.09%
	2	Feb. 12–14	38.43 ± 2.28%	77.38 ± 9.99%	7.15 ± 1.21%	7.12 ± 0.85%
	3	Feb. 19–23	30.52 ± 2.34%	52.08 ± 4.31%	15.22 ± 2.81%	12.52 ± 1.92%

per cent as compared with 36.1 per cent for the groups of ex-conjugants from clone *E*40*a*. There can be no doubt that conjugation regularly results in a very much greater mortality in the clone *E*81*a* than in the clone *E*40*a*.

Turning now to an examination of the relations between the values obtained for the ex-conjugants and the values obtained for the non-conjugants of the two races, the pertinent data will be found in Tables XIII and XIV, the former giving the mortality data, the latter giving the ratios of means, standard deviations, and coefficients of variation.

With one exception the ratios of ex-conjugant to non-conjugant mean fission rates in *E*40*a* are very close to 1.0, whereas the ratios in *E*81*a* vary from 0.33 to 0.61. The single exception in the case of *E*40*a* is in the second experiment. Observations in this experiment were discontinued after three days because of a defect in the culture method which resulted in great reduction in fission rates, and extraordinarily high mortality. For this reason, the data of this experiment are of doubtful value. The ratios in the other experiments were quite consistent: 1.14, 1.04, and 0.90 for *E*40*a*; 0.54, 0.61, and 0.48 for *E*81*a*. It therefore appears that, under favorable conditions, conjugation regularly makes little or no difference in the mean fission rate of *E*40*a*, but regularly reduces to about one-half the mean fission rate of *E*81*a*.

Table XIII

Comparison of ex-conjugants and non-conjugants of clones E40a and E81a. Percentage mortality.

Experiment	Period	Clone *E*40*a*			Clone *E*81*a*		
		Ex-conj.	Non-conj.	Ex-conj. minus non-conj.	Ex-conj. minus non-conj.	Ex-conj.	Non-conj.
1	Jan. 12–25, 1931	12.5%	0 %	12.5%	43.8%	81.3%	37.5%
2	Feb. 9–14, 1931	14.3%	0 %	14.3%	40.9%	40.9%	0 %
3	Feb. 15–28, 1931	71.9%	75.0%	−3.1%	18.2%	99.5%	81.3%
4	Mar. 18–Apr. 1, 1931	47.5%	40.0%	7.5%	22.1%	92.9%	70.8%

The ratios of standard deviations show no consistent differences between the two clones. On the other hand, the ratios of coefficients of variation are consistently higher in *E*81*a* than in *E*40*a*: 4.18 as compared with 1.16; 1.69 as compared with 1.00; 10.87 as compared with 5.38; 4.16 with 2.01. It is thus clear that conjugation regularly increases relative variability in the race *E*81*a* much more than it does in *E*40*a*. Within either race, the magnitude of the increase varies considerably in different experiments, but within any one experiment the relative variability of *E*81*a* is increased more by conjugation than the relative variability of *E*40*a*. This is clearly due to the fact that conjugation greatly lowers the mean fission rate (without correspondingly reducing the standard deviation) in clone *E*81*a* but does not lower mean fission rate in clone *E*40*a*.

Conjugation also increases mortality by a greater amount in *E*81*a* than in *E*40*a*. In the first experiment the mortality of *E*40*a* is increased by conjugation from zero per cent to 12.5 per cent; a difference of 12.5 per cent; the mortality of *E*81*a* is increased from 37.5 per cent to 81.3 per cent, a difference of 43.8 per cent. In the second experiment, *E*40*a* increased from zero per cent to 14.3 per cent, a difference of 14.3 per cent; *E*81*a* increased from zero per cent to 40.9 per cent, a difference of 40.9 per cent. In the third experiment, *E*40*a* decreased from 75.0 per cent to 71.9 per cent, a decrease of 3.1 per cent; *E*81*a* increased from 81.3 per cent to 99.5 per cent, an increase of 18.2 per cent. In the fourth experiment, *E*40*a* increased from 40.0 per cent to 47.5 per cent, an increase of 7.5 per cent; *E*81*a* increased from 70.8 per cent to 92.9 per cent, an increase of 22.1 per cent.

TABLE XIV

Comparison of clones E40a and E81a in the ratios of ex-conjugants to non-conjugants in mean total number of fissions, standard deviation and coefficient of variation

Experiment	Period	Mean total number of fissions		Standard deviation		Coefficient of variation	
		Clone *E*40*a*	Clone *E*81*a*	Clone *E*40*a*	Clone *E*81*a*	Clone *E*40*a*	Clone *E*81*a*
1	Jan. 16–20	1.14	0.54	1.32	2.27	1.16	4.18
	Jan. 21–25	1.04	0.61	1.04	1.03	1.00	1.69
2	Feb. 12–14	0.60	0.33	3.21	3.59	5.38	10.87
3	Feb. 19–23	0.90	0.48	1.81	1.99	2.01	4.16

Thus repeated comparison of the effects of conjugation within the two clones *E*40*a* and *E*81*a* confirms the diversity in effects of conjugation shown by the two clones in the experiments reported in Section IV and shows that differences of the same character occur at each conjugation. In clone *E*81*a* conjugation regularly decreases fission rates; while in clone *E*40*a* it does not. In clone *E*81*a* conjugation regularly increases the relative variability more than it does in *E*40*a* (this is a consequence of the decrease in mean fission rate produced in *E*81*a*). Further, in *E*81*a*, conjugation regularly increases the mortality greatly, while in *E*40*a* it increases the mortality but slightly.

Conjugation between *the Clones E81a and E40a*

In view of the decided differences between these two clones, it appeared of great interest to attempt cross conjugation between them. This was done and twenty pairs of conjugants were obtained in which one member of each pair was from the clone *E*40*a*, the other member of the pair from the clone *E*81*a*. The ex-conjugant lines from these 40 individuals were compared with 40 ex-conjugants of *E*40*a* and with 40 ex-conjugants of *E*81*a*. The results of this experiment, together with the results of crosses between other diverse races, will be reported in detail later in a paper devoted exclusively to the subject of cross-conjugation. At this point, however, it may be stated that ex-conjugants of the cross between *E*40*a* and *E*81*a* differed markedly from non-conjugants of both clones and from ex-conjugants of both clones.

This result confirms the results previously elaborated in detail, namely, that the effects of conjugation on mean fission rate, on variability, and on mortality depend on the genetic constitution of the individuals which conjugate.

VI. SUMMARY OF RESULTS AND GENERAL CONCLUSIONS

Summary of results:

Part I.—Three conjugations within the clone 247*a*:

1. Conjugation had no effect on mean fission rate in this clone.
2. Conjugation increased both standard deviation and cœfficient of variation of fission rate.
3. Conjugation had no consistent effect on mortality in this clone.

Part II.—Simultaneous conjugations within six ex-conjugant clones derived from the clone 247*a*:

1. In mean fission rate the ex-conjugants of one of the clones, *E*81*a*, are consistently and significantly lower than ex-conjugants of any of the other five clones.
2. In standard deviation, the ex-conjugants of clone *E*81*a* are consistently higher than those of all the other clones; those of *E*40*a* are consistently lower than all others; those of *E*41*a*, *E*46*b*, and *E*80*b* are consistently intermediate.
3. In cœfficient of variation, the same significant consistent gradation of the groups exists as was found with respect to standard deviation.

4. In mortality, the ex-conjugants of clones *E*40*a* and *E*41*a* are very low, those of *E*81*a* very high, those of *E*46*b*, *E*80*b* and *E*85*b* intermediate.
5. It was demonstrated that the differences between the ex-conjugants of the clone *E*81*a* and those of the other clones were not due to self-perpetuating differences in cultural conditions; they were due to constitutional differences between this clone and the others.
6. Differences in mean fission rate between ex-conjugant groups parallel differences in mean fission rate between the non-conjugant groups from which they were derived.
7. The clone *E*80*b* had its mean fission rate very slightly increased by conjugation; the clone *E*41*a* had its mean fission rate unaltered by conjugation; the clone *E*81*a* had its mean fission rate reduced about 50 per cent by conjugation.
8. Conjugation increased absolute variability in fission rate about twice as much in the clone *E*41*a* as in the clone *E*40*a*.
9. Conjugation also increased the relative variability of fission rate about twice as much in the clone *E*41*a* as in the clone *E*40*a*.
10. Conjugation did not significantly increase variation in clone *E*40*a*, but did markedly increase it in clone *E*41*a*.
11. Conjugation produced practically no change in mortality in clones *E*85*b* and *E*80*b*, but it greatly increased mortality in the clone *E*81*a*.

Part III.—Four comparisons of the effects of conjugation in the clones *E*40*a* and *E*81*a*.

1. The distribution of fission rates among the ex-conjugants of the two clones are consistently different.
2. The mean fission rates of *E*40*a* ex-conjugants are consistently and significantly higher than the mean fission rates of *E*81*a* ex-conjugants.
3. The two groups of ex-conjugants do not differ consistently in standard deviation.
4. The cœfficients of variation of the *E*81*a* groups of ex-conjugants are always greater than those of the *E*40*a* groups of ex-conjugants.
5. The mortality of *E*81*a* groups of ex-conjugants is always greater than the mortality of *E*40*a* groups of ex-conjugants.

6. Conjugation regularly leaves the mean fission rate of *E*40*a* unaltered, but regularly decreases markedly the mean fission rate of *E*81*a*.
7. Conjugation in these two clones produces no uniform differences in its effect on standard deviation.
8. Conjugation regularly increases the coefficient of variation in *E*81*a* more than in *E*40*a*.
9. Conjugation regularly increases mortality more in *E*81*a* than in *E*40*a*.
10. A group of cross-conjugants between the two diverse clones *E*81*a* and *E*40*a*, was found to differ from groups of non-conjugants and from groups of inbred conjugants of either one of the parent clones. (Details to be given in later paper.)

General Conclusions:

1. Conjugation increases fission rate in some clones, decreases fission rate in others; it increases variation in some clones, but (at least, under some conditions) not in others; it increases mortality in some clones, but not in others.
2. There are characteristic diversities between certain clones in the effect of conjugation on mean fission rate, variability, and mortality. These can be demonstrated repeatedly.
3. The physiological effects of conjugation are not the same in all clones of even a single species, but depend on the constitution of the individuals which conjugate, and differ when the constitutions of the conjugating individuals differ.
4. The diverse effects of conjugation found to be characteristic of different clones of *P. aurelia* bear an interesting relation to the diverse effects of conjugation (and the various theories of conjugation based on these diverse effects) reported by various investigators: Diversities similar to those reported to be characteristic of different species are here found to be characteristic of different clones of a single species. It is therefore suggested that the disagreement in results and theories of previous investigators may to some extent be due to failure to examine the effects of conjugation in a sufficient number of different races within each species. In *P. aurelia*, at least, results in agreement with each of the two leading theories of conjugation may be obtained by studying an appropriate race.

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THE EFFECTS OF X-RAYS ON FERTILITY IN DROSOPHILA MELANOGASTER TREATED AT DIFFERENT STAGES IN DEVELOPMENT

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Introduction

Despite the numerous investigations in which *Drosophila* has been subjected to X-ray treatment, little is known concerning the effects which irradiation produces upon fertility in this fly. In 1927 Mavor employed the egg, larval, pupal, and adult stages of the fruit fly to determine the dosage of X-ray lethal to dipteran protoplasm. In 1926 and 1927 Packard reported the effects of X-rays upon protoplasm based upon the mortality of *Drosophila melanogaster* when subjected to X-ray treatment. In the treatment of mature males of *Drosophila*, Muller (1928) observed a high frequency of sterility in the adult offspring of the treated flies. Further work approaching the problem of the effects of irradiation upon fertility was accomplished by Hanson (1928). In this work mature male *Drosophila* were exposed to X-rays and the effects upon the mortality of eggs, larvæ and pupæ observed. A later report, and one more closely associated with fertility, is that of Hanson and Ferris (1929) upon fecundity in *Drosophila* exposed to irradiations.

A review of the literature, together with results obtained in a preliminary experiment in which eggs, larvæ and adults of both sexes were treated with X-rays, suggested that variations in fertility might be attributed to the variety of developmental stages of germ cells present at the time of treatment. This in turn suggested the problem of determining the effects of irradiation upon fertility when germ cells in different stages of development were subjected to X-ray treatment.

Material and Methods

In order to secure different stages of germ cells for treatment, wild type males were mated to wild type females. The flies were mated in vials which contained a small paper spoon filled with food. Females were allowed to deposit eggs for a period of 12 hours, after which the spoons were removed and others containing fresh food inserted into the vials. These second spoons were allowed to remain in the vials for an additional 12 hours. Thus, all eggs deposited on the food were 0–12 hours old at the time of removal from the culture tubes. Egg bearing food from the spoons was placed in culture dishes to await the proper age for treatment. A portion of the eggs were held for 24 hours after the laying period was completed before treatment was given, a second portion for 72 hours; flies from a third portion were treated 30 hours after hatching and a fourth portion furnished males and females for the control series. Males and females from each group were separated upon emergence and held until nine days after treatment before they were mated. All cultures were kept at a temperature of 26.5° C. during development and all, except the controls, were given an equal amount of irradiation. The dosage of X-rays employed was that known as D5 (1325 *r* units), material being placed twelve centimeters from the target and the machine operated for twelve and one-half minutes at 50 kv. and 10 ma. using a one millimeter aluminum screen.

Matings for testing the fertility of the irradiated and control flies were made in vials containing spoons filled with food. In the case of treated individuals, a treated fly was mated to an untreated individual and if no eggs were deposited within two days after mating, the untreated individual of the pair was replaced by a second untreated fly. This precaution was deemed necessary to avoid errors due to the sterility of the untreated mate. Wild males and wild females were mated as controls.

Eggs were collected for a period of 8 days or until 100 eggs were secured from each mating. The spoons were removed from the tubes every twelve hours, the eggs counted under a binocular and the material transferred to food bottles where the eggs completed their development. Eggs were considered fertile only when they produced imagoes. Adults emerging from the eggs deposited by (or in the case of males fertilized by) an individual fly were counted and the percentage of fertility arrived at by dividing the number of adults by the original number of eggs in the culture. To obtain the percentage of fertility displayed by a group of flies, the mean fertility of the group was calculated by the formula

$$M = (a - c)i,$$

where a is an assumed mean, c is a correction factor and i the class interval, M being corrected by the probable error of the standard deviation of the group.

To determine the duration of the effects of irradiation upon fertility and sterility each group of flies was kept supplied with fresh food until twenty-five days had elapsed after the date on which they were treated with X-rays. During this time both males and females (the same pair used in the 9 to 17-day period) were allowed to remain in the mating tubes. Beginning with the twenty-fifth day after irradiation eggs were collected over a period of 8 days, or until 100 eggs were obtained from each mating. Since a few of the treated flies failed to survive until the beginning of the second period, the comparisons of effects shown by the first collection of eggs (9 days after treatment) and those found in the second collection (25 days after treatment) involve slightly fewer flies than were considered in calculating the initial effects. To obtain figures of statistical value concerning the permanency of effects of irradiations upon fertility and to determine whether or not induced sterility was permanent, comparisons were made of the performance of only those flies carried through both the 9-day period and the 25-day period.

A word as to the meaning of the terms *sterility* and *fertility* as employed herein seems to be in order. Those experimental females, or females mated to experimental males, which deposited no eggs during the course of the laying period, or which produced eggs from which no adult flies emerged, are considered sterile. The term *fertility* applies only to those cases where a part, or all, of the eggs deposited developed into imagoes and is a measure of the number of eggs that succeeded in completing development.

Experimental Results

Sterility

The experiments reported in this paper provide data concerning the effects of irradiations upon sterility and fertility as defined above and throw some light upon the duration of these effects. Table I is a composite of the results upon the fertility of eggs deposited and the sterility produced. From this table it may be seen that five of the thirty-five control males were sterile, or 14.3 per cent. The same percentage of control females proved to be sterile. Of twelve males, treated as adults, two were sterile, or 16.66 per cent. Comparing this figure of sterility to that found for the control males we find a difference of 2.3 ± 8.09 per cent,—a difference too small to be significant. Treatment of larvæ at the ages of 72–84 hours rendered 33.33 per cent of the males sterile, while of males from larvæ treated at the ages of 24–36 hours, 50 per cent were found to be incapable of reproduction. The percentage of sterility found in the group of males derived from larvæ treated at 72–84 hours of age differs from that of the control group by 19.05 ± 8.29 per cent. Males obtained from larvæ treated at 24–36 hours of age differ in sterility from controls by 35.72 ± 6.7 per cent.

TABLE I

A Table Showing the Fertility of Flies 9–17 Days after X-ray Treatment: Dosage D5

Age at treatment	Number treated	Sex	Number fertile	Number sterile	Percentage sterile	Difference in percentage of sterility	Eggs laid	Flies	Average percentage of fertility	Difference in percentage of fertility
Adult	12	♂	10	2	16.66	2.3 ± 8.09	776	380	44 ± 5	45 ± 5.2
72–84 hr. larvae	15	♂	10	5	33.33	19.05 ± 8.29	1080	648	58 ± 5.2	28 ± 5.4
24–36 hr. larvae	20	♂	10	10	50	35.72 ± 6.7	923	502	52 ± 2.3	34 ± 2.8
Adult	15	♀	10	5	33.33	19.05 ± 9.1	915	641	70 ± 1.0	16 ± 1.8
72–84 hr. larvae	11	♀	10	1	9.0	−4.19 ± 7.8	1000	714	71 ± 3.4	15 ± 3.8
24–36 hr. larvae	17	♂	10	7	41.7	26.89 ± 8.3	933	756	81.3 ± 2.5	4.7 ± 2.5
Control	35	♀	30	5	14.3	—	2979	2571	86 ± 1.5	—

The sterility found in the various groups of females parallels that observed in males treated at corresponding ages. When females were treated as adults 33.33 per cent of the individuals were sterile. Subtracting the percentage of sterile controls from this figure leaves a difference of 19.05 ± 9.1 per cent. The group of females from larvæ irradiated at the ages of 72–84 hours contained very few sterile flies,—one in eleven, or 9.0 per cent. Here the percentage of sterile individuals was less than in the control group. However, it may be seen that a high degree of sterility maintained among females from larvæ treated at 24–36 hours of age. In this group 41.7 per cent of the individuals were sterilized. The sterility in this case differs from that found in control females by 26.89 ± 8.3 per cent, the difference being 3.24 times the probable error.

Table II

A Table of Calculations

Comparison of the fertility of treated and control flies

Percentage of fertility in controls	Percentage of fertility in treated	Difference in percentage	$\frac{\text{Difference}}{\text{probable error}}$
86 ± 1.5	Adult ♂ 44 ± 5.0	42 ± 5.2	8.07
86 ± 1.5	72–84 hr. ♂ 58 ± 5.2	28 ± 5.4	5.2
86 ± 1.5	24–36 hr. ♂ 52 ± 2.3	34 ± 2.8	12.1
86 ± 1.5	Adult ♀ 70 ± 1.0	16 ± 1.8	8.9
86 ± 1.5	72–84 hr. ♀ 71 ± 3.4	15 ± 3.8	3.94
86 ± 1.5	24–36 hr. ♀ 81.3 ± 2.5	4.7 ± 2.5	1.92

Comparison of fertility in sex of treated flies

Percentage of fertility	Percentage of fertility	Difference in percentage	$\frac{\text{Difference}}{\text{probable error}}$
Adult ♀ 70 ± 1.0	Adult ♂ 44 ± 5.0	26 ± 5.8	4.9
72–84 hr. ♀ 71 ± 3.4	72–84 hr. ♂ 58 ± 5.2	13 ± 6.2	2.1
24–36 hr. ♀ 81.3 ± 2.5	24–36 hr. ♂ 52 ± 2.3	29.3 ± 3.4	8.6

Fertility of Eggs Deposited

The table of calculations (Table II) was devised to compare the fertility of treated groups to the fertility displayed by the control series. From the data on control flies we find the mean fertility of the group to be 86 ± 1.5 per cent. When adult males were treated with irradiations, the mean fertility of eggs subjected to fertilization by them was 44 per cent with a probable error of ± 5 per cent. This group of treated males differs in fertility from the controls by 42 ± 5.2 per cent, the difference being 8.7 times its probable error. Males treated in larval stages exhibit no such marked reduction in ability to fertilize eggs as do males irradiated after metamorphosis. The fertility of eggs collected from matings of males X-rayed at 72–84 hours of age amounts to 58 per cent of all eggs secured from the group, the probable error being ± 5.2 per cent. The mean fertility of this group is found to differ from that of the controls by 28 ± 5.4 per cent; a difference 5.2 times the probable error. The fertility of males treated at the larval ages of 24–36 hours is slightly lower than that of males irradiated when 72–84 hours old. Of 923 eggs collected from females crossed to males that were X-rayed at 24–36 hours of age, 52 ± 2.3 per cent produced adults. This figure (52 ± 2.3 per cent) subtracted from the fertility displayed by controls (86 ± 1.5 per cent) leaves the significant difference of 34 ± 2.8 per cent.

Thus far the fertility of treated males only has been considered. A review of the data obtained from treated females, Table II, shows that eggs of females, irradiated thirty hours after emerging from the pupal stage, exhibit a mean fertility of 70 ± 1.0 per cent. A comparison of the egg fertility of this group to that of the control group reveals that, due to the effects of irradiation, 16 ± 1.8 per cent of the eggs from females treated as adults failed to complete their development. This figure, 8.9 times its probable error, is highly significant. The mean fertility of eggs from females treated as 72–84 hour larvæ was 71 ± 3.4 per cent, or 15 ± 3.8 per cent less than that of controls. However, treatment of 24–36 hour larvæ reduced the fertility of eggs deposited by females from such larvæ by only 4.7 ± 2.5 per cent. Since this figure is only 1.92 times its probable error no significance can be attached to it.

Permanence of Effects upon Fertility and Sterility

The volume of data on the permanence of the effects of irradiation upon fertility is such as to exclude it from this publication and a brief review of the findings is included in the section on discussion.

From a study of the data contained in Table III it may be seen that in some cases flies which were fertile at 9 days after treatment became sterile within 25 days after irradiation. Nine such cases appear among 52 treated individuals. Of 14 flies found sterile at 9 days after treatment, 3 regained their fertility within 25 days, or 21.4 per cent of the flies were temporarily sterilized by irradiations. On the other hand, 6.66 per cent of the control flies found fertile 9 days after mating were sterile in the 25-day period, while none of the controls that were sterile

Table III

A Table Comparing the Sterile Flies of Two Periods

	9–17 days after treatment			25–33 days after treatment		
Serial Number	Eggs	Flies	Sterile	Eggs	Flies	Sterile
A ♂ 6	83	2	—	100	0	1
A ♂ 9	7	3	—	37	0	1
A ♀ 6	100	39	—	56	0	1
A ♀ 13	0	0	1	0	0	1
A ♀ 14	0	0	1	7	0	1
A ♀ 15	0	0	1	21	0	1
C ♂ 10	59	10	—	0	0	1
C ♂ 11	0	0	1	100	89	—
C ♂ 13	45	0	1	6	0	1
C ♂ 14	0	0	1	100	55	—
C ♂ 15	0	0	1	6	0	1
C ♀ 2	100	82	—	24	0	1
B ♂ 4	55	1	—	9	0	1
B ♂ 7	100	88	—	36	0	1
B ♂ 10	100	2	—	3	0	1
B ♂ 15	0	0	1	24	0	1
B ♂ 16	0	0	1	0	0	1
B ♂ 18	0	0	1	0	0	1
B ♂ 19	29	0	1	38	0	1
B ♀ 8	50	42	—	1	0	1
B ♀ 12	6	0	1	27	0	1
B ♀ 14	36	0	1	0	0	1
B ♀ 17	18	0	1	11	3	—
Total	788	269	14	606	147	20
Controls						
D 24	79	33	—	38	0	1
D 31	100	0	1	100	0	1
D 32	100	0	1	100	0	1
D 33	100	0	1	100	0	1
Total	379	33	3	338	0	4

9 days after mating produced offspring during the 25-day period of mating.

Discussion

Little more can be said by way of discussing the sterility effects of irradiation than to point out the percentage differences between the treated and control groups. It is unnecessary to apply a mathematical formula to determine that there is no difference in the sterility of males treated as adults and that of the control males. A larger percentage of sterility is found if males derived from larvæ irradiated at 72–84 hours of age are tested. In this group the percentage of sterile individuals differs from that of the controls by 19.05 ± 8.29 per cent, a figure not mathematically significant but perhaps suggestive. However, males obtained from larvæ treated at 24–36 hours of age differ in sterility from controls by 35.72 ± 6.7 per cent. In this case there is no doubt that X-rays produced sterile individuals, since the difference is 5.33 times the probable error.

Analysis of the data on females shows the sterility of only one group to differ significantly from the sterility found for the control females. The percentage of sterile flies found among females from larva X-rayed at 24–36 hours of age differs from that of the control females by 26.89 ± 8.3 per cent. Therefore, in the case of either males or females taken from larvæ treated at 24–36 hours of age, irradiation produces sterile individuals. It is also quite probable that X-rays produced sterility in the other treated groups tested, but the dosage used was too light and the numbers were too small for obtaining definite proof.

In normal stocks of *Drosophila* the proportion of eggs which hatch at room temperature is remarkably constant and the percentage of fertile eggs deposited is extremely high. Packard (1927) reported an average fertility of 97 per cent, basing his report upon the number of larvæ appearing in the cultures. The thirty fertile controls used in this experiment deposited 2979 eggs, 86 per cent of which completed metamorphosis. When we consider that Packard used a different strain of flies and based the fertility upon larvæ rather than adults that emerged, this figure compares very favorably with the fertility which he observed.

Table II compares the fertility of the treated groups to the fertility displayed by the control series. When the mean fertilities of the different groups of treated males are compared to the fertility of eggs obtained from controls, we find the differences ranging from 42 ± 5.2 per cent, if males treated as adults are compared, to 28 ± 5.4 per cent, if male larvæ irradiated at 72–84 hours of age are used. It appears at once that the reductions of fertility effected by irradiations are significant. Clearly, the action of X-rays upon the germ cells of the male rendered a large proportion of those cells incapable of entering into the fertilization process, or produced effects within the sperm cells which inhibited the development of the zygotes of which such cells have become a part.

From the experimental data it is obvious that the fertility of males as defined in this paper is reduced by the action of X-rays irrespective of the developmental stage irradiated. The highest degree of fertility displayed by any of the three groups of males tested represents a reduction in fertility of 28 ± 5.4 per cent, while the greatest reduction of 42 ± 5.2 per cent is evidence of the extreme effects of X-rays upon the fertility of males treated during adult life. These statements are, however, to be regarded, not as facts which must recur since the difference between any two groups of treated males (the greatest difference being 14 ± 7.2 per cent, or 1.94 times the probable error) is so small as to be insignificant. In case these suggestions point in the right direction, as the data indicate, it is well to note that only the fertility of eggs is considered, sterile flies being eliminated. This might be called the " net " fertility. If we consider the sterile flies produced in the various groups plus the egg fertility, opposite results are obtained. That is to say, the " gross " fertility of adult males is affected less than that of males treated in the larval stages.

Going briefly into the findings of the fertility of irradiated females, we see that the fertilities of only those females from larvæ treated at 72–84 hours of age and the females irradiated as adults differ significantly from that of the control group. Treatment of 24 to 36-hour larvæ reduced the fertility of eggs deposited by females from such larvæ by only 4.7 ± 2.5 per cent. Since this figure is only 1.92 times its probable error, we cannot conclude that irradiation reduces the fertility of eggs if the females are treated at the larval age of 24–36 hours. This result may be due to the severe action of X-rays, which produces sterile individuals in a large number of the cases in which the gonads are affected, or to an interruption of developmental processes which inhibits the appearance of the individual whose gonads are affected by the absorption of radiant energy. Be that as it may, the data show clearly that the fertility of females treated in the later stages of development is reduced by the action of irradiations.

It is interesting to note that there is no significant difference in the egg fertility observed if the different groups of treated males are compared, or if the three groups of irradiated females are compared with one another. On the other hand, if we compare the fertility of males to the fertility of females treated in the same stage of development, we find in each case, with one exception, that the fertility of eggs is more affected by treating males than by treating females. The fertility of treated adult males is 26 ± 5.8 per cent lower than that of treated adult females. This figure is 4.5 times the probable error and without doubt is significant of an inherent difference in the germ cells of the sexes which accounts for the greater susceptibility of the sperm cells to irradiations. The males and females irradiated at the larval age of 72–84 hours show a difference in fertility of 13 ± 6.2 per cent, favoring the females, or a figure 2.1 times its probable error, which is slightly indicative of a greater reduction in the fertility of males than in the fertility of females. The fertility of the males derived from larvæ treated earlier in life (24–36 hours) differs greatly from that of the females. In this case the fertility of the males is 29 ± 3.4 per cent lower than the fertility of females.

Summarizing the effects of X-rays upon the fertility of flies treated at different stages of development we may say, if sterile flies are eliminated, that (1) except by treatment of female larvæ 24–36 hours of age, the fertility of either sex may be reduced by X-ray treatment irrespective of the age at which the flies are irradiated, (2) the effects of irradiation upon the fertility of female *Drosophila* are at a minimum when 24 to 36-hour larvæ are treated, (3) there is no significant difference in the fertility of a sex, regardless of the age at which the flies are treated, but (4) it is evident that if treatment is administered to opposite sexes of *Drosophila* in the same stages of development, X-rays of the dosage used affect the fertility of males to a greater degree than that of females, irrespective of the age at which the flies are subjected to treatment.

It is curious to note that a dosage of X-rays considerably higher than that here used had been found by Muller to have the opposite effect,—reducing the fertility more in adult females than in adult males.

Two questions arise: Are the effects which irradiations produce upon fertility of a permanent nature? Is sterility, once induced, to remain a characteristic of the individual throughout life, or is such sterility of a temporary nature? To determine the answers to these important and interesting questions two periods of egg collection were observed. These were fully described above as the 9 to 17-day period and the 25 to 33-day period and will be referred to as such in the following discussion.

Comparisons of average fertilities found for the groups during the 9 to 17-day period with those found for the 25 to 33-day period appear at first glance to indicate that eggs deposited in the second period exhibited a higher degree of fertility than eggs deposited in the first period. Considering the number of flies included in these calculations it is hardly advisable to depend upon a comparison of the fertilities exhibited by the groups during the two periods. The numbers are so small and the variations in the fertility of the individual flies so great that the difference in fertility exhibited by one or two flies may influence the average percentage of the group in such manner as to make it slightly higher for the 25 to 33-day period, whereas had these few flies varied as the remainder of the group the average would have been lower for the 25 to 33-day period than for the 9 to 17-day test. But if the fertilities exhibited by the individual flies at nine days after irradiation are compared to those exhibited twenty-five days after treatment, it becomes evident that some of the flies were more fertile in the second than in the first period, while others were less fertile in the second period than in the first. In either sex, irrespective of the stage in development at which the flies were treated, the fertility displayed twenty-five days after irradiation was often different from that exhibited nine days after the administration of X-rays. Although it is certain that the fertility of untreated flies decreases with age, we cannot say whether the fertility of irradiated individuals will increase or decrease with the passage of time. We can, however, say with some degree of certainty that the percentage of fertile eggs deposited, or fertilized by treated flies, varies greatly at different dates after irradiation. After X-ray treatment the rate of fertilization is highly variable irrespective of the sex which has been subjected to irradiations. In other words, a large number of eggs deposited by treated flies during one period may be viable, while of those collected at some other date relatively few may be capable of development.

From a close study of the data included in Table III we find a few instances in which flies were fertile at nine days after treatment but became sterile within twenty-five days after irradiation. Of the fertile treated flies 17.3 per cent became sterile between the seventeenth day and the twenty-fourth day following X-ray treatment. Of fourteen flies sterile at nine days after treatment, three regained their fertility within twenty-five days, 21.4 per cent of the sterile flies were only temporarily so. There is, perhaps, no significant difference in the percentage of flies regaining fertility and the percentage becoming sterile, but significance may be attached to the fact that some flies sterilized by X-rays were found to become fertile with the passage of time following irradiation while none of the four flies found sterile among the controls ever became fertile. On the other hand, 6.66 per cent of the control flies found fertile nine days after mating were sterile in the 25-day period, indicating a natural tendency for normal flies to become sterile with age.

From the second period of egg collection (25–33 days after treatment of flies, or after mating controls) we learn that (1) the percentage of fertile eggs deposited at nine days and twenty-five days after irradiation is not constant, (2) flies may be rendered temporarily sterile by X-ray treatment, but sterile untreated flies fail to become fertile with time and (3) untreated flies decrease in fertility with age.

It seems reasonable to conclude that the effects of irradiation upon the fertility of *Drosophila melanogaster* are not permanent, and that fertilization may be effected rather irregularly by treated flies. Certainly, since flies are temporarily sterilized by X-rays, the action of irradiations in producing sterile individuals is not always permanent.

The author wishes to express thanks to Professor J. T. Patterson and to Professor H. J. Muller for their many helpful suggestions during the course of the experiments.

Conclusions

1. With the moderate dosage used, the degree of fertility exhibited by *Drosophila* eggs is more affected by the treatment of males than by the irradiation of females of the same age, regardless of the stage in development at which irradiations are administered. Females from cultures of larvæ treated at 24–36 hours of age display, when fertile at all, a higher degree of fertility than flies of either sex, irrespective of the age at which they are irradiated.

2. Irradiation of larvæ 72–84 hours of age sterilizes a smaller proportion of the flies of either sex than treatment of any other larval stage, and less sterility is found among females when larvæ are treated at this age than in any other group of flies tested.

3. When larvæ only are irradiated more males are sterilized than females.

4. The effects of irradiation upon fertility are not permanent. Irradiated flies are variable in the degree of fertility which they exhibit at different dates after treatment. Fertility may increase with the passage of time following treatment, but the fertility of control flies decreases with age.

5. The action of X-rays in producing sterile flies may often have only a temporary effect.

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THE EFFECT OF TEMPERATURE ON THE LEG POSTURE AND SPEED OF CREEPING IN THE ANT LASIUS

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Introduction

The present paper is an attempt to investigate the influence of temperature upon the locomotion of the ants, *Lasius niger* and *L. umbratus*. In particular it deals with the correlation between temperature and leg-spread, and temperature and speed.

Shapley has determined the correlation between temperature and speed of progression for several species of ants. His first paper (Shapley, 1920) reported that, for *Liometopum apiculatum*, " . . . an empirical curve is obtained that for any temperature throughout a range of 30° centigrade gives the speed with an average probable error of 5 per cent for one observation. Conversely, from a single observation of the ant-speed, the temperature can be predicted within 1 degree centigrade."

In his second paper (1924), Shapley applied the Van't Hoff-Arrhenius equation to his data. He stated: "A constant value of μ at about 11,000, for temperatures from 20° C. to 35° C., appears to be justified. But further observation is necessary to test the indicated variability of μ for temperatures below 20° C." Assuming a break at 20° C., he approximates μ as 20,000 for the lower temperatures.

Hoagland (1931) has studied the influence of temperature on learning in ants, but this does not concern us here.

Wheeler's book (1913) may be consulted for an account of the ecological aspect of the influence of temperatures on ants. Fielde (1905), in particular, has observed the effect of temperature on the development of ants.

Leg-spread and Temperature

The relationship between leg-spread and temperature was determined as follows: Ants were permitted to crawl over smoked kymograph paper, lying in a covered Petri dish. The dish was placed in either a dark, constant-temperature chamber, or in a glass vessel immersed in the water of a thermostat. In the latter case, light was eliminated by the addition of India ink to the water.

The records were then shellacked, after which microphotographs were made, with the helpful advice of Professor A. Petrunkevitch.

The trails made by the metathoracic legs are easily seen and studied on the microphotographs (Fig. 1). Specimens of *L. umbratus,* taken from a genetically homogeneous colony, were utilized. In all cases it was found that as the temperature rose, the legs were held further apart. Since the increase in leg-spread per degree rise in temperature is very small, the phenomenon is best illustrated over a fairly large temperature difference.

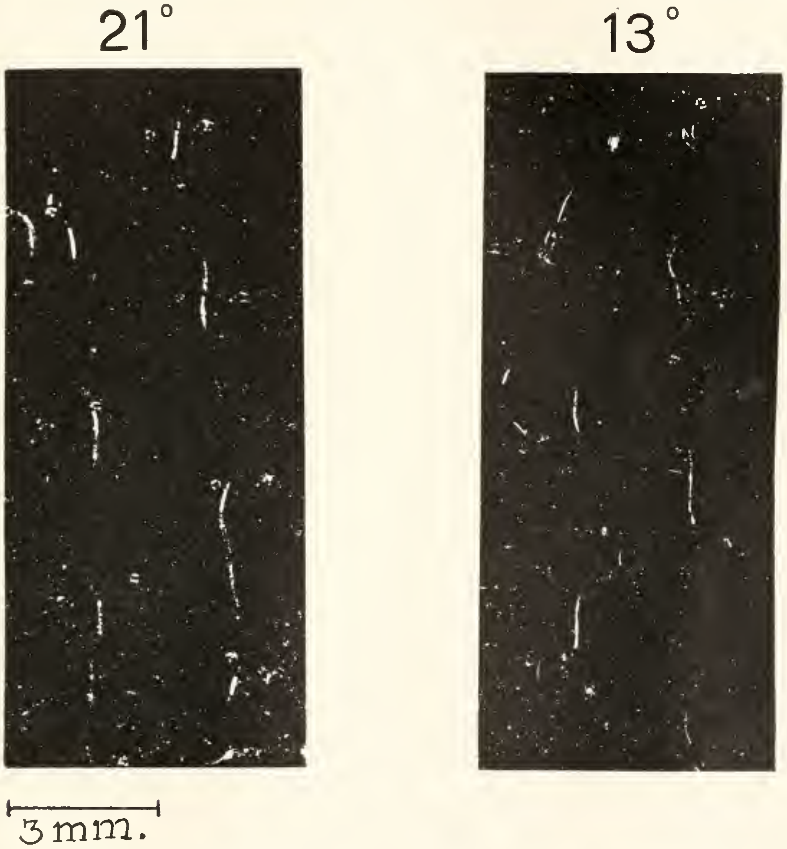

FIG. 1. Microphotographs of the footprints of *Lasius umbratus* (No. 124) on smoked kymograph paper. These records were taken in the dark at 21° and at 13° C. after one-half hour for thermal adjustment. The greater leg-spread at 21° is evident but is more striking over a wider temperature difference. The long streaks are made by the hind legs. These photographs are not re-touched.

A typical instance of this phenomenon is demonstrated by Specimen 124 (Figs. 1 and 2). At 12° C. the leg-spread was 2.36 ± .31 mm.; at 21° C., 2.81 ± .20 mm.; at 25° C., 2.88 ± .23 mm.

SPEED AND TEMPERATURE

Methods

The thermostat consisted of a large glass jar filled with water. The temperature was regulated by a mercury toluene contact and by the addition of ice or hot water. Bubbles of compressed air thoroughly stirred the system.

The ants crawled in a glass tube 15 cm. long, of 5 mm. internal diameter, on which lines were etched at 5 cm. intervals. The ends of this tube were lightly plugged with moist cotton and were attached by rubber tubing to spirals of glass tubing, the free ends of which extended out of the thermostat. Thus air adjusted to a given temperature could circulate through the system. This we think necessary since ants died when left overnight if the 15 cm. glass tube was corked at both ends, but survived if it was lightly plugged with cotton.

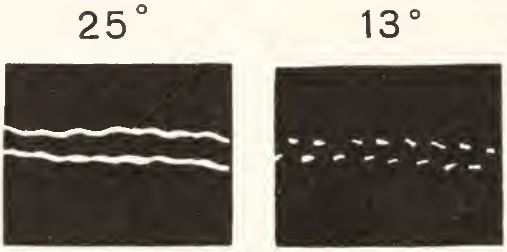

FIG. 2. Trails made by *Lasius umbratus* crawling over smoked kymograph paper; the actual tracings are covered with white paint to demonstrate the width of the paths. Upper tracing ant No. 124, 25° C. Lower tracing, same individual at 13°. At the higher temperature the hind legs are further apart and with many specimens make an unbroken tracing: see text.

A thermometer placed in the 15 cm. glass tube indicated that about ten minutes was necessary for the air within the tube to reach the same temperature as the surrounding water.

Actively crawling ants were taken from the two genetically homogeneous formicaries of *L. umbratus* and *L. niger,* placed in the 15 cm. glass tube, and, after an interval of at least ten minutes, were clocked with a stop watch as they crawled between the 5 cm. lines. When necessary the tube was gently tapped to induce crawling. Actively crawling specimens were used because observations over a long period indicate a rhythm of activity and rest in the colony as a whole.

A diffuse light of approximately 25-foot candles flooded the entire thermostat. We do not regard the light conditions as a disturbing factor.

Data

The data accumulated for *L. umbratus* are graphed in Fig. 3. The ordinates represent the log of the rate of progression:

$$\frac{1}{\text{time in seconds for 5 cm.}} \times 100.$$

The abscissæ are the reciprocals of the absolute temperatures. These units were used so that the data could be applied to the Van't Hoff-Arrhenius equation.

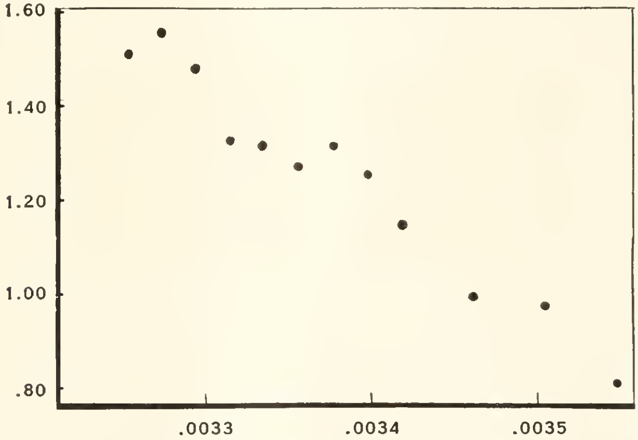

FIG. 3. Rate of creeping as controlled by temperature in *Lasius umbratus.* The log of the progression rate, $\frac{1}{\text{time in secs. for 5 cm.}} \times 100$, is plotted against the reciprocal of the absolute temperature (abscissæ). Each point is the average of 8–20 stop watch readings. Because of the scattering of points, no certain line could be drawn.

Each point on the graph represents from 8 to 20 readings. The temperature range is limited by the facts that below 10° C. it is very difficult to induce crawling, while above 30° C. the specimens are injured, some dying at the latter temperature.

The graph clearly shows that the speed of progression is influenced by temperature. On the basis of centimeters per second, the increase is from .3 cm. per second (average) at 10° C., to 1.6 cm. per second at 24° C. Sample time records for 5 cm. runs are shown in Table I.

The value of μ, the energy of activation of the catalyst, in the Van't Hoff-Arrhenius equation, was called the temperature characteristic by

TABLE I

Rate of locomotion. Mean time, based on 8 trials, ant crawling 5 centimeters in each.

Temperature	Individual	Time
°*C.*		*seconds*
12	120 (umbratus)	11.6
18	114 (umbratus)	4.3
16	97 (niger)	7.1
20	97 (niger)	5.5
25	99 (niger)	2.8

Crozier (1924), when he applied the equation to biological phenomena. The form of the equation we used was:

$$\ln \frac{R^2}{R^1} = \frac{\mu}{2}\left(\frac{1}{T^1} - \frac{1}{T^2}\right)$$

R^1 is the rate at T^1, and R^2 is the rate at T^2. T is the absolute temperature. In using the equation a line is drawn through the graphed points, and the rates are then calculated from it.

In Fig. 3 it will be noticed that there is a greater scatter of points in the higher range. Furthermore, since the lower range also exhibits scattering, we do not put too much faith in the rectilinear relation. However, we have calculated a value of μ for it, which is 22,500 calories.

The data accumulated for *L. niger* are graphed in Fig. 4. The linear relationship between temperature and speed is evident, and a sharp break at 20° C. is easily seen. From 15° to 20° C. μ has a value of 10,700

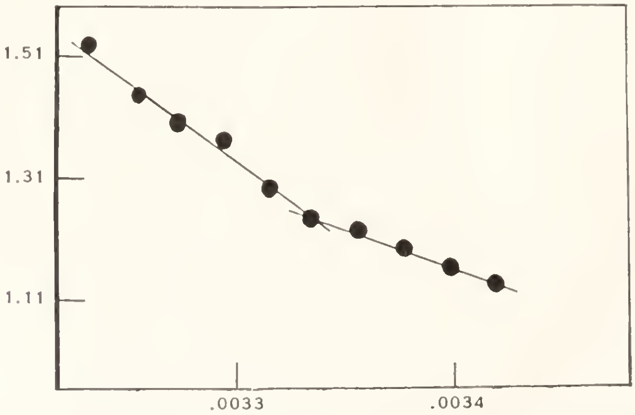

FIG. 4. Rate of creeping as controlled by temperature in *Lasius niger*. Abscissæ and ordinates on same basis as in Fig. 3. Note the clear-cut break at 20° C., the critical temperature.

TABLE II

Values of μ *(Temperature Characteristic Determined)*

Species	Activity	Temperatures ° *C.*	μ
L. umbratus	Speed of progression	10–19	22,500
L. umbratus	Frequency of leg movements	12–25	12,700
L. niger	Speed of progression	16–20	10,700
L. niger	Speed of progression	20–25	22,900

calories; from 20° to 25° C., a value of 22,900 calories. Sample time records are shown in Table I.

The foregoing values of μ are based on speed, which itself must be dependent in part on the frequency of leg movement—a difficult quantity to determine. Several attempts to do this, however, were made. The method was simply to observe the ant in the thermostat and clock the time for ten steps of the metathoracic leg. At low temperatures this is not difficult, but in the higher range the frequency determined cannot be very accurate. *L. umbratus,* Specimen No. 128, showed the following mean frequencies per second, based on 7 readings for each temperature: 12° C., 2.13; 18° C., 3.45; 25° C., 5.55. This gives μ the value of 12,700 calories.

DISCUSSION

In the case of *L. umbratus* we have experienced difficulty in fitting a curve to the experimental data for speed of progression, so that we might utilize the Van't Hoff-Arrhenius equation. Our data plainly show that speed of progression increases as the temperature rises, as do leg-spread and frequency of leg movement. Furthermore, leg-spread and frequency of leg movement no doubt largely determine speed of progression. But each of these three has its own rate of increase. We desire to point out that the locomotion of ants involves a number of complex factors.

Crozier and Stier (1925) have shown that in the tent caterpillar speed of progression is not a simple exponential function of $1/T_{\text{abs}}$, but that frequency of abdominal peristaltic locomotor waves, a factor of the former, is, giving the value of 12,200 calories.

Crozier (1925) pointed out that the value for μ of 12,200 calories in arthropods has been found for various rhythmic types of neuro-muscular activity (respiratory excepted). These include speed of progression of a diplopod, the calls of the katydid and cricket, and the flashing of the firefly. Also, our colleague Professor G. Evelyn Hutchinson (1928) has found a μ of 11,500 for the speed of progression of an interesting South African isopod, *Preatoicus capensis.*

Crozier's μ's for Shapley's 1920 *Liometopum* data were 25,900 for below 16° C., and 12,200 for from 16° to 38° C. Our findings roughly approximate these, except in the case of *L. umbratus,* as can be seen in Table II. It should be remembered that the data for *speed* of progression in *L. umbratus* are none too good a basis for the calculation of μ.

SUMMARY

1. In the progression of *L. umbratus* the leg-spread and the frequency of leg movements increase as the temperature rises.

2. The speed of progression as influenced by temperature in *L. niger* shows a critical temperature at 20° C., and yields two values for μ: 10,700 between 16–20° C., and 22,900 between 20–25° C.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

IV. Is the Permeability of the Erythrocyte to Water Decreased by Narcotics?

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I

In the course of studies connected with his well-known " Haftdruck " theory, Traube (1908) made the observation that certain lipoid-soluble substances, such as amyl alcohol, which are also known to possess narcotic properties, may, under appropriate conditions, exert an antihemolytic effect. This effect he interpreted as being due to " eine Verdickung der Lipoidschicht welche die Stabilität der Blutkörperchen gegen andere Hämolytika ändern muss." A few years later Arrhenius and Bubanović (1913) obtained similar results with chloroform, ethyl ether, ethyl and amyl alcohols, and benzene. As an example of these results one typical experiment with a hypotonic solution containing chloroform may be cited. In this experiment it was found after one hour at 37° C., followed by several more in the ice-box, that the percentage of hemolysis decreased from 60 per cent in the absence of chloroform to a minimum of 37 per cent in the presence of 0.2 per cent, rising again at higher concentrations. Arrhenius and Bubanović did not hesitate to conclude that " diese Wirkung beruht vermutlich auf einer Verlangsamung des Eindringens von Wasser in die Zellen." Among the more recent workers in this field, Yoshitomi (1920) found after exposures of 2 to 18 hours to hypotonic solutions, a lesser degree of hemolysis in the presence of certain concentrations of ether, chloroform, chloretone and amylene hydrate than in their absence. He suggested that this effect might be due either to a less ready entrance of water into, or a more ready escape of salts from, the cells. Jarisch (1921) also obtained an inhibition of osmotic hemolysis with alcohol, ether, amylene hydrate and urethane. While he did not state in very precise terms his conception of the antihemolytic action of these substances, his use in this connection of the term " wasserhemmend " would seem to indicate that his views were not unlike those of Arrhenius and Bubanović.

Since these and similar results obtained with the erythrocyte have frequently been cited in support of the view that narcosis is associated with a decreased cell permeability, it seems necessary for us to point out that such evidence is entirely inconclusive. In the first place, osmotic hemolysis is a complicated process involving not only (*a*) the entrance of water into the erythrocyte, but (*b*) the escape of hemoglobin, (*c*) the possible loss from the cell of salts and other osmotically active substances (see in this connection Ponder and Saslow, 1931) as well as (*d*) a variety of possible changes of diverse nature, not directly associated with permeability, in what is commonly and rather loosely called the "osmotic resistance" of the cell. While it is undoubtedly true that anything that decreases the permeability of the cell to water (factor *a*) will, in general, tend to delay osmotic hemolysis, the reverse statement is by no means true. A delay might equally well be produced by the operation of any or all of factors *b, c*, and *d*.

A more fundamental objection, however, to evidence of the type mentioned above, is that it is very unlikely that the investigators in question obtained from their experiments any real information as to the *rate* of hemolysis, which is the thing of greatest importance in connection with questions of permeability to water. It is, of course, conceivable that if at the end of some single arbitrarily selected time a lesser degree of hemolysis is obtained in the presence than in the absence of a narcotic, the difference might be due to a slower rate of progress in the former case towards the same final end state of the system. On the other hand, the possibility must be considered that the narcotic exerts its effect primarily on the degree of hemolysis ultimately attained; in that case, with no knowledge of the position of equilibrium towards which the system is proceeding—or which it may indeed have reached at the time the observation is made—no valid conclusions whatever can be drawn as to the fundamental rate of the hemolytic process.

Though this principle would seem to be a self-evident one, it has been very frequently disregarded in the past, not merely in studies on hemolysis, but in other fields of physiological work as well. It should be most strongly emphasized, therefore, that while it is possible to study a position of equilibrium with no exact knowledge of the rate at which it is attained, the reverse procedure of attempting to draw conclusions concerning a rate, with no information whatever as to the end state which the given system is approaching, is entirely unwarranted and can lead only to confusion (see in this connection Jacobs, 1928). In the present paper the distinction between the effects of certain urethanes on the "equilibrium" and the "rate" factors concerned in osmotic hemolysis will be illustrated; and it will further be pointed out that since as far as we are aware the work of previous investigators in this field makes no such distinction, it can be expected to throw no real light on the question of the permeability of the erythrocyte to water.

II

As a first step in the separation of the two types of factors, it seemed important to obtain curves representing the entire course of hemolysis from its beginning until further change had ceased. This necessary type of information, which, as has been mentioned, has apparently not been supplied by previous workers, is particularly easy to obtain by the method of one of the authors (Jacobs, 1930). A typical experiment is represented in Fig. 1 in which the erythrocytes were those of the ox and the narcotic was ethyl urethane. The blood in this, as in all the other experiments here described, was thoroughly and almost instantaneously mixed with the solutions used in the proportion of approximately 1 to 500. Kymograph records were made of the ensuing hemolysis and from these the curves in the figure were reconstructed. Because of the enormous importance of pH and temperature changes in experiments involving equilibria, the precautions described by Jacobs and Parpart (1931) were employed in all such experiments, the pH being kept almost constant in this case at approximately 7.4, and the temperature at 20° C.

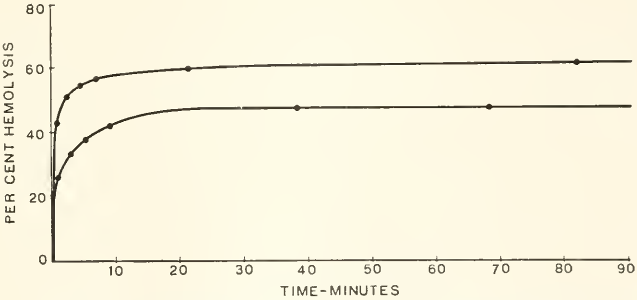

FIG. 1. Course of hemolysis of ox blood in buffered 0.090 M NaCl in the presence (lower curve) and absence (upper curve) of 0.3 M ethyl urethane: pH, 7.4; temperature, 20° C.

An inspection of Fig. 1 shows very clearly that under the conditions of this experiment the effect of ethyl urethane upon the final end-point reached by the system is far more striking than any possible effect it might have upon the rate of hemolysis as such. Thus, in the absence of the urethane, a degree of hemolysis of approximately 61 per cent was attained in between 20 and 30 minutes, and after that time no further change occurred; in its presence a final degree of hemolysis of 48 per cent was reached in about the same time, and this likewise underwent no further change. Very similar results were obtained in a number of other experiments. It is evident, therefore, that no information about the rate of hemolysis can be secured in such cases by observing at the end of some arbitrarily selected time the mere degree of hemolysis that then happens to exist.

It is to be noted that the antihemolytic effect of ethyl urethane is not a simple osmotic one due to the greater total concentration of the solution containing it. Such an effect could be obtained only with a non-penetrating substance, since otherwise the solute molecules would distribute themselves inside and outside the cell in such a way that their osmotic effects would everywhere balance. In the case of the urethanes, however, not only is the penetrating power for cells in general known to be extremely high, but a number of hematokrit measurements made in the course of these experiments showed the absence of any measurable osmotic effects on cell volume. As a matter of fact, though the concentration of the ethyl urethane in this particular experiment was 0.3 M, the antihemolytic effect was only that which would have been produced osmotically by an increase in concentration of possibly 0.006 M in a solution of a non-penetrating non-electrolyte.

As to the effect of ethyl urethane on the fundamental rate of hemolysis, apart from that on the final degree attained, this cannot be determined by a mere inspection of such curves as those in Fig. 1. Even though a measurably longer time were required to attain a given degree of hemolysis in the presence than in the absence of the narcotic, it would be impossible to be certain, without a fairly complicated mathematical analysis of the results, how much of the observed effect was due merely to the shift in the final equilibrium. Since, therefore, even under the relatively favorable conditions provided by the possession of two complete hemolysis curves it is very difficult to draw conclusions about the effect of a narcotic on the rate of the process, what information of value could conceivably be obtained from the knowledge of only a single point on each curve? As a matter of fact, most of the observations mentioned in the introductory paragraph were taken at times long after those at which the final equilibrium must have been attained and could, therefore, by no possibility throw any light upon the rate of hemolysis, and by implication, upon the possible rate of entrance of water into the cells. Though, as will be shown later, the conclusion of previous investigators that the rate of entrance of water into the erythrocyte is slowed by the presence of a narcotic may in itself be entirely correct, it may not validly be drawn from the data they have presented.

Having found in experiments of the type of that represented in Fig. 1 that the final equilibrium condition is usually reached within 20 or 30 minutes, and in any case in less than an hour, it seemed desirable to study by more quantitative methods than those previously used the effects of varying the concentration of the narcotic. In Table I are given the results of one such experiment with ethyl urethane in which the percentages of hemolysis reached in one hour in the presence of different concentrations of this substance were determined. The concentration of NaCl used was selected as a favorable one for the particular sample of ox blood employed. The pH in this case was approximately 7.35 and the temperature, as before, 20° C. It will be noted that as the concentration of urethane increased, the degree of hemolysis decreased until a maximum effect was reached probably somewhere between 0.25 M and 0.5 M. Beyond this point a hemolytic effect of the narcotic became evident. The general results of this entirely typical experiment with ethyl urethane do not differ in principle from those obtained by Arrhenius and Bubanović with chloroform, if it be assumed, as was almost certainly the case, that what these investigators measured was the final position of equilibrium of the system.

TABLE I

Degree of hemolysis attained by ox erythrocytes in one hour in a buffered 0.085 M NaCl solution containing different concentrations of ethyl urethane.

pH, 7.35; temperature, 20° C.

Concentration of urethane	Percentage of hemolysis
—	75
0.0078	75
0.0156	74
0.0313	74
0.0625	70
0.125	70
0.25	68
0.5	73
1.0	100

In another experiment, whose results are given in Table II, the general procedure was reversed by keeping the concentration of urethane constant at 0.1 M and varying that of the hypotonic solution. In this way the antihemolytic effect of the narcotic on a considerable proportion of all the erythrocytes in the blood could be observed. It will be noted that although the results show certain minor irregularities, there is in no case any departure from the previously observed antihemolytic effect. Similar, though less complete, results were also obtained with several other urethanes, as well as with ethyl alcohol. Taking together these results and those reported by previous workers, the evidence seems to be entirely consistent that the degree of hemolysis ultimately attained in a hypotonic solution may be reduced by a variety of narcotic substances in proper concentrations. This fact, however, though of interest in other ways, throws little or no light upon the question of the effect of narcotics upon the permeability of the erythrocyte to water. What is needed is information not about the final equilibrium but about the fundamental rate at which hemolysis occurs.

III

It has been pointed out in another place by one of the authors (Jacobs, 1928) that in cases where the position of equilibrium of a hemolytic system is influenced by the same factor whose effect on the rate of hemolysis it is desired to study, the general rule should be followed of keeping the system at all times as far away from equilibrium conditions as possible. Thus, in investigating the effect of temperature upon the rate of osmotic hemolysis, it was found that where only water or very strongly hypotonic solutions were employed consistent and plausible results could be obtained, while with less strongly hypotonic solutions the results were erratic and at first sight inexplicable. Similarly, in the case of narcotics, which have been shown in the preceding section to affect in a striking manner the degree of hemolysis finally attained, the only satisfactory method of studying their effect on the rate

TABLE II

Degree of hemolysis attained by ox erythrocytes in one hour in buffered NaCl solutions of different concentrations in the absence and presence of 0.1 M ethyl urethane.
pH, approximately 7.35; temperature, 20° C.

Concentration of NaCl	Percentage of Hemolysis	
	Urethane absent	Urethane present
0.098	82	78
0.099	78	74
0.100	73	69
0.101	71	65
0.102	69	59
0.103	67	55
0.104	63	53
0.105	57	48
0.106	53	44
0.107	50	37
0.108	45	38
0.109	41	33
0.110	34	22

of hemolysis as such would be to work with very strongly hypotonic solutions, or preferably, with distilled water. Most methods for studying hemolysis are much too slow for use in experiments of this type—which doubtless accounts for the fact that they have apparently not heretofore been made. The method of one of the authors (Jacobs, 1930) is entirely suitable for this purpose, however, and some results obtained with it may now be described.

In Table III are indicated the effects on hemolysis by water of the addition of different amounts of ethyl, n-butyl, i-amyl and phenyl urethanes. Though for such short times the experimental errors are relatively large, it will be noted that the results are on the whole entirely consistent and that for each substance there is a slight but unmistakable retardation of hemolysis as the concentration increases. Furthermore, the effectiveness of the different urethanes, as might be expected, proves to be very different. As judged by the dilutions at which a retardation of hemolysis first becomes apparent, the order of effectiveness is:

TABLE III

Effect of Various Urethanes on the Time Required for 75 Per Cent Hemolysis of Ox Blood by Distilled Water

Ethyl Urethane		n-Butyl Urethane		i-Amyl Urethane		Phenyl Urethane *	
Concentration	Time	Concentration	Time	Concentration	Time	Concentration	Time
	seconds		*seconds*		*seconds*		*seconds*
—	1.5	—	1.3	—	1.30	—	1.25
0.0313	1.5	0.0015	1.4	0.0003	1.30	0.00025	1.25
0.0625	1.5	0.0031	1.4	0.0013	1.28	0.0005	1.30
0.125	1.7	0.0062	1.5	0.0025	1.32	0.001	1.30
0.25	2.0	0.0125	1.6	0.005	1.42	0.002	1.35
0.5	2.2	0.025	1.5	0.01	1.50	0.004	1.32
1.0	2.6	0.05	2.1			0.008	1.52

* See also Table V.

$$\text{ethyl} < \text{n-butyl} < \text{i-amyl} < \text{phenyl}.$$

This is not only the order in which narcotic effects are usually manifested by these substances, but there is a rough quantitative agreement between the concentrations at which hemolysis begins to be retarded and those found by Dr. E. B. Harvey to be effective in reversibly suppressing the cleavage of the *Arbacia* egg. The latter concentrations, as cited by Lucké (1931), are as follows: ethyl, 0.05 M to 0.2 M; n-butyl, 0.0125 M to 0.05 M; i-amyl, 0.005 M to 0.01 M; and phenyl, 0.00125 M to 0.005 M.

In the case of ethyl urethane, where the effective concentration is fairly high, it is conceivable that at least a part of the delay in hemolysis may be due to osmotic factors. Even a substance that penetrates a cell as rapidly as a urethane might, if sufficiently concentrated, slow to a measurable extent the rate of attainment of the final osmotic equilibrium between the cell and its surroundings, while having no direct effect upon the position of the equilibrium. Whatever may be the validity of this objection in the case of ethyl urethane, however, it is certain that it cannot hold in the case of the other three substances, where the effective concentrations are of the order of 0.01 M to 0.001 M. It has been shown elsewhere by one of the authors (Jacobs, 1932) that even in the case of a completely non-penetrating non-electrolyte such as saccharose, the time required for hemolysis in a 0.01 M solution differs from that in water only to a barely measurable extent, the difference for ox erythrocytes being perhaps 0.1 second. With lower concentrations than 0.01 M of substances of extremely high penetrating power such as the urethanes, direct osmotic effects could certainly not be measured; and any effects that could be measured would therefore necessarily be of a more specific nature. Though the retardation of hemolysis in water by urethanes is apparently always small in amount and the errors of the determinations are relatively large, the results obtained in these and in other experiments have been sufficiently consistent to leave little doubt that the substances in question are able to affect the rate of hemolysis under conditions where any shift in the final theoretical equilibrium is of negligible importance. Such an effect, though erroneously inferred by other workers from their experiments, has not, we believe, previously been demonstrated.

TABLE IV

Times required for 75 per cent hemolysis of ox blood in NaCl solutions containing different amounts of ethyl urethane. Temperature, 20° C.

Concentration of Urethane	Concentration of NaCl							
	Water	0.02 M	0.04 M	0.06 M	0.07 M	0.073 M	0.076 M	0.08 M
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
——	1.5	4.0	5.7	7.0	10.3	18.0	22.5	70.2
0.0313 M.....	1.5	4.2	5.6	7.5	11.7	19.8	26.8	73.4
0.0625 M.....	1.5	4.0	5.8	7.3	12.5	18.9	34.4	96.5
0.125 M......	1.7	4.0	6.2	8.3	16.0	27.8	56.5	156
0.25 M.......	2.0	4.7	6.5	9.1	20.3	54	103.5	332
0.5 M........	2.2	4.8	7.0	9.7	39.0	140	280	832
1.0 M........	2.6	5.1	7.1	10.3	32.6?	274	317	420

In concluding the presentation of experimental data there may be added in Tables IV and V figures showing the gradual transition from conditions where the fundamental rate factor is primarily concerned in determining the time of hemolysis to those where the equilibrium factor tends to dominate the situation. In the light of the facts presented in the preceding section, it is evident that the striking effects produced by all the substances in the most concentrated salt solutions are due chiefly to a change of the hemolytic end-point in the direction of a reduced final degree of hemolysis. The rate factor as such cannot be studied in such solutions. In the case of ethyl urethane, one additional factor appears in the last figure of the last column, namely, a direct hemolytic effect of the urethane in very high concentrations; but this factor is of comparatively small importance in connection with the present experiments.

TABLE V

Times required for 75 per cent hemolysis of ox blood in NaCl solutions containing different amounts of phenyl urethane. Temperature, 20° C. Each figure for water and for the lower concentrations of NaCl is the average of four determinations.

Concentration of Urethane	Concentration of NaCl						
	Water	0.02 M	0.04 M	0.06 M	0.075 M	0.08 M	0.085 M
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
——	1.50	3.87	5.17	6.92	11.1	19.8	41
0.0005 M	1.50	—	5.17	6.70	11.8	28.5	46
0.001 M	1.55	3.88	5.23	6.72	12.8	30.0	55
0.002 M	1.52	3.85	5.20	7.05	14.5	76.5	115
0.004 M	1.70	3.90	5.30	7.58	48.5	86.0	—
0.008 M	1.95	3.93	5.38	7.95	54.2	121.0	870

One other point suggested by the results obtained with i-amyl and phenyl urethanes may be mentioned briefly, namely, that whereas the retarding effect of urethanes on hemolysis by water never failed to appear in our experiments, it was sometimes more doubtful in the case of the most dilute salt solutions, *e.g.*, 0.02 M. The experiment chosen for representation in Table V was selected to show this condition, which was, however, not invariably observed. We are unable to suggest a reason why the retarding effect of certain urethanes may at times be more pronounced in the absence than in the presence of salts, but may point out that a somewhat similar but much more striking effect has been observed by Lucké (1931) in the case of the *Arbacia* egg. His explanation is that if the entrance of water into the cell has already been strongly retarded by the presence of salts (especially those of calcium), narcotics are unable to produce any further effect. In the case of the erythrocyte it will be shown in a later paper that very low concentrations of salts, including NaCl, are effective in some non-osmotic manner in retarding osmotic hemolysis. It is conceivable, therefore, though by no means certain, that a similar principle is involved in the two cases.

IV

It has been shown in the preceding sections that various urethanes in the proper concentrations are able to reduce the degree of hemolysis finally attained in certain hypotonic salt solutions. This increase in the osmotic resistance of the cells, which has been noted by a number of previous observers, is an equilibrium rather than a rate effect and is best seen in solutions whose concentrations are such as to cause the disappearance of some but not all of the erythrocytes in the given sample of blood. It has also been shown that under conditions where complete hemolysis is very rapidly produced and where possible changes in the theoretical position of equilibrium of the system are of negligible importance, narcotics are able to bring about a slight but consistent slowing of the rate of the process. This effect, which is best seen in distilled water and which has frequently been confused with the one first mentioned, has not as far as we are aware, previously been demonstrated, though from the standpoint of cell permeability it is the more important of the two. Its possible nature will be considered after attention has first been given to the more striking and better known change in the osmotic resistance of the cells.

As has been mentioned above, there are at least four different ways in which the osmotic resistance of the erythrocyte might be affected (factors *a, b, c* and *d* on page 314). Of these factors, the first, namely, a change in permeability to water, may almost certainly be ruled out as a possible cause of any change in the position of final equilibrium of the hemolytic system. Only if the cells at some point became completely impermeable to water could this factor alone do more than change the rate at which the equilibrium is reached; the equilibrium itself would remain unaltered. As a matter of fact there is an abundance of evidence that the erythrocytes do not at any time become completely impermeable to water.

On the other hand, a changed permeability to salts and other osmotically active substances contained within the cell (factor *c*) might conceivably alter its osmotic resistance. Since hemolysis by hypotonic solutions is due to an excess of osmotic pressure within the cell, any escape of materials that reduced this excess would not only slow the rate of hemolysis in all cases, but in certain critical cases would prevent its occurrence altogether. The possibility of a leakage of salts from the erythrocyte in ordinary osmotic experiments has been emphasized by Ponder and Saslow (1931), and it is by no means inconceivable, or indeed unlikely, on *a priori* grounds that such a leakage might be favored by narcotics. In sufficiently high concentrations these substances tend by destroying the erythrocyte to permit a very ready escape of materials from its interior; and it is entirely possible that in lower concentrations they might injure its surface sufficiently to increase any loss of electrolytes already in progress.

Though this theory has a certain degree of plausibility, it nevertheless seems necessary to discard it in view of the direct evidence obtained by Siebeck (1922) that narcotics actually reduce to an easily measurable extent the rate of exchange of ions between the cell and its surroundings, and that furnished by Joel (1915) that the gradual increase in the electrical conductance of a suspension of erythrocytes is slowed rather than accelerated in the presence of such substances. As far as the available evidence goes, the effect of ordinary concentrations of narcotics would seem, if this factor were of importance, to be in the direction of reducing rather than of increasing the osmotic resistance of the erythrocyte.

Turning next to factor *d*, which involves some change or changes in the osmotic resistance of the cell not associated with permeability factors, the possibility suggested by Traube that the narcotic may produce in some way a thickening or a strengthening of the cell membrane and so oppose hemolysis may first be considered. Such an explanation appears to be an unlikely one in view of the fact that the surface of the erythrocyte seems normally to offer little resistance to osmotic volume changes. (See in this connection Jacobs 1931, 1932.) As a matter of fact, the increased resistance in the presence of, for example, 0.3 M ethyl urethane, which is by no means the greatest effect we have observed, may correspond to a change in the critical concentration of NaCl by 0.003 M, amounting in terms of osmotic pressure to perhaps one-eighth of an atmosphere. That the delicate cell membrane could be strengthened to support this excess of pressure does not seem very likely.

A much more plausible possibility is that the narcotic may in some way have a tendency to cause a diminution in the volume of the cell and so to oppose its swelling in hypotonic solutions. Effects of this sort are already known in the case of other agents. For example, the increased osmotic resistance of erythrocytes in alkaline media and at high temperatures (Jacobs and Parpart, 1931) and in solutions of non-electrolytes (Jacobs, 1932) is probably to be accounted for in this way. As a matter of fact, v. Knaffl-Lenz (1918) has reported a decrease in the volumes of erythrocytes, as measured by the hematokrit, on the addition of certain narcotics, though the times required to produce this effect in his experiments were much longer than those involved in the present series; and, furthermore, his results were indecisive in the case of the only urethane he used. We have been unable to detect with certainty by the hematokrit method any such differences in volume in the case of ethyl urethane solutions, though in view of the rather large errors of the hematokrit method and the very slight volume changes required to produce a considerable difference in the observed percentage of hemolysis (Jacobs and Parpart, 1931) we do not feel that this possibility has been entirely ruled out.

The last factor that will be discussed is the second of those mentioned above, namely, the escape of hemoglobin from the cell. This factor has frequently been neglected in studies on osmotic hemolysis in the past owing, no doubt, to the old belief that hemolysis is produced by an actual bursting of the cell when the internal pressure has reached a sufficiently high point. If this were the mechanism of hemolysis, then the escape of hemoglobin would, in fact, be an unimportant part of the process. It seems certain, however, from the phenomenon of "reversible hemolysis," so-called, that the cell is not ordinarily ruptured by mild hemolytic agents, but that at a certain time, as a result of stretching or some other change in its surrounding membrane, the latter becomes permeable to the hemoglobin contained within the cell. This permeability to hemoglobin is reached in such a sudden and definite manner that osmotic hemolysis is apparently an "all-or-none" phenomenon, *i.e.*, up to a certain point no hemoglobin escapes from the cell; beyond that point an almost infinitesimal increase in the volume of the cell results in the free outward diffusion of all of its hemoglobin (Saslow, 1929; Parpart, 1931). Unfortunately, we know too little at present about the physical state of the hemoglobin within the cell and the possible effects of changes in this state on its diffusibility. It is usually assumed, however, in the absence of evidence to the contrary, that under all usual conditions we have to do with a simple aqueous solution of hemoglobin and that the possibility of its escape from the cell depends merely on the character of the cell membrane.

The assumption that the escape of hemoglobin from the erythrocyte depends primarily on the cell membrane may or may not be true. It is of interest, however, to see whether it can be made the basis of a plausible explanation of the effect of narcotics on osmotic resistance. There is considerable evidence that at the surface of the erythrocyte in addition to lipoid substances which give to the cell certain of its physical properties (Mudd, S., and E. B. H. Mudd, 1926) and which perhaps determine its free permeability to all lipoid-soluble substances, there are regions through which water, ions, and non-lipoid-soluble organic substances of low molecular weight can pass. Though the exact structural nature of these regions is not known, they may, at least in a semi-figurative sense, be called "pores." A further discussion of this theory as applied to the erythrocyte is given by Mond and Hoffman (1928) and by Jacobs (1931).

Whatever may be our ideas of the exact nature of the hypothetical "pores" in the cell membrane, it must not be forgotten that certain purely objective facts are well known; namely, that non-lipoid-soluble molecules of sufficiently low molecular weight pass through the wall of the erythrocyte readily, those of higher molecular weight more slowly, and those whose molecular weight (or molecular volume) exceeds a certain size fail to do so at all. The hemoglobin molecule, of course, enormously exceeds the critical size for penetration. Nevertheless, in osmotic hemolysis a point is somewhere reached where rather suddenly the cell becomes permeable to hemoglobin. Without attaching too literal a meaning to the statement, we may say that at this point the "pores" have been enlarged sufficiently to permit the escape of this molecule.

Now we have a certain amount of experimental evidence that narcotics are able—presumably by adsorption—to diminish the size of the pores in artificial membranes, or, at any rate, to render more difficult the passage of certain substances through these membranes (Anselmino, 1928 *a, b*). Suppose that the same were true of the erythrocyte at the point where it undergoes hemolysis. In this case, the presence of a sufficient concentration of a narcotic substance might be expected to convert a "pore" that would otherwise just permit the passage of hemoglobin into one that would just fail to permit it. Further swelling would be necessary to cause hemolysis. The osmotic resistance of the cell would thereby be raised, just as it is known to be in fact. Furthermore, the effectiveness of weakly adsorbed narcotics would be less than that of strongly adsorbed ones and, again, there is a parallel between the adsorbability of different urethanes and their ability to prevent hemolysis.

Accepting in a purely tentative manner this explanation of the effect of narcotics upon the final equilibrium of a hemolytic system, how would such an explanation fit the known facts concerning the rate at which hemolysis occurs in very strongly hypotonic solutions? It is entirely conceivable that in such solutions the rate of osmotic hemolysis might be affected either by a slowing of the rate of entrance of water or by a slowing,—though not a prevention—of the escape of hemoglobin by a delay in the attainment of the proper condition of the pores, or by a combination of both factors. Since it is unlikely, with a rate of increase of cell volume as rapid as that in distilled water, that the delay in the escape of hemoglobin would be very great, it seems entirely possible that at least a part of the observed effect of narcotics on the rate of hemolysis by water may be due to an actually decreased rate of penetration of this substance. It is to be noted, however, that the possible effect must in any case be rather slight.

In this connection it is of interest to consider the work of Siebeck (1922) on the effect of narcotics on the rate of passage of ions between the erythrocyte and its surroundings and also that of Anselmino and Hoenig (1930) on the entrance of the non-electrolytes erythritol, arabinose, xylose, etc. In the former case, actual chemical analyses were made at several intervals and it is therefore virtually certain that the permeability of the cell to the substances in question was dealt with directly. In the work of Anselmino and Hoenig, though the methods were not quite so direct, it is also very likely that their interpretation of their results as indicating a production by narcotics of a decreased permeability to various slowly penetrating non-electrolytes is correct. It is perhaps significant that the decrease in permeability to ions and to rather slowly penetrating non-electrolytes is much greater than any decrease for water that could be inferred from the present experiments. If the " pore " theory were correct, it would be expected that the hypothetical diminution of the pore diameter produced by narcotics would exert an effect upon permeability which would become proportionately greater as the size of the molecule increased. The water molecule, being the smallest of those commonly supposed to enter the erythrocyte in this manner, would be affected least of all.

It should be emphasized that this explanation of the manner in which narcotics may conceivably affect osmotic hemolysis is suggested merely as a convenient working hypothesis. Its chief advantages are that it explains in essentially the same manner both " rate " and " equilibrium " effects and that, as far as we are aware, it is not incompatible with any known facts. It is by no means necessary, however, that the rate and equilibrium effects should be explained in the same way; in the case of temperature, for example (Jacobs, 1928), they seem almost certainly to be of a different nature. It is entirely possible that at any time facts may come to light with which the present theory is inconsistent; in that case it may readily be abandoned without greatly changing the significance of the experimental data here presented.

We are glad to acknowledge our indebtedness to Dr. Balduin Lucké for supplying most of the urethanes used in this work, and to Ethel R. Parpart and G. E. Shattuck for assistance in connection with several of the experiments.

SUMMARY

1. The observation of previous investigators that narcotic substances in proper concentrations tend to oppose osmotic hemolysis is confirmed in the case of several urethanes.

2. It is shown that the conclusion frequently drawn from such observations, that the antihemolytic effect of narcotics is due to a decreased permeability of the erythrocyte to water, is unwarranted by the existing experimental evidence. The necessity for a separation of " rate " and " equilibrium " factors in studies on osmotic hemolysis is emphasized.

3. It is shown by experiments in which these factors are properly separated that a slight but measurable retardation of osmotic hemolysis may be produced by low concentrations of urethanes. The possible nature of the mechanism of this retardation, which may perhaps in part involve a decreased permeability of the cell to water, is discussed.

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THE LIFE HISTORY OF PARORCHIS AVITUS (LINTON) A TREMATODE FROM THE CLOACA OF THE GULL

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The study of parasitic flatworms in the United States began with Joseph Leidy, who described a number of larval and adult trematodes. For many years progress consisted in the description of new species of adult flukes. More recently, attention has been directed to a study of the larval stages and at present one hundred and sixty-six cercariæ have been described in the United States. Only twenty of these larvæ, however, have been definitely correlated with their adult forms. It is obvious therefore that further advance is dependent upon knowledge of the life history of these species. Lack of such information keeps the literature burdened with almost twice as many specific names as are necessary, since each larval trematode whose adult stage is not recognized, is described as a distinct species. Furthermore, and of more significance, knowledge of the successive stages in development is indispensable for correct identification, taxonomy, and any fundamental work on the physiology and control of these parasites. The fact that fifteen of the twenty life histories solved in this country have been described in the last four years indicates the intensity of present interest in life history studies. This paper adds another to the list of known life histories and describes more completely the results reported by the writers in a recent preliminary note (Stunkard and Cable, 1931).

Experiments conducted at the Marine Biological Laboratory during the summer of 1931 have shown that *Cercaria sensifera* (Stunkard and Shaw, 1931) is the larva of *Parorchis avitus* (Linton, 1914). *Parorchis avitus* was originally described from the cloaca of the herring gull, *Larus argentatus.* It is very similar to *Parorchis acanthus*, a species described by Nicoll (1906) from the bursa and cloaca of *Larus argentatus* and first given the name *Zeugorchis acanthus.* Subsequently Nicoll (1907*a*) erected the genus *Parorchis* to supplant *Zeugorchis* (preoccupied) and named *P. acanthus* as type species.

Parorchis acanthus has been the subject of important studies by English investigators. Nicoll (1907*b*) gave a more complete description of the species and reported its occurrence in the common gull, *Larus canus.* In the same work he transferred *Distomum pittacium* Braun, 1902 to the genus *Parorchis.* Nicoll, in a letter to Linton, expressed the belief that *Parorchis avitus* is probably identical with *P. acanthus.* Linton (1928) however, showed that *P. avitus* differs from *P. acanthus* in the same respects that *P. acanthus* differs from *Parorchis* (*Distomum*) *pittacium*, namely, the ratio of sucker diameters and the extent of uterine convolution. Lebour (1914) compared young stages of *P. acanthus* with *Cercaria purpuræ* Lebour, 1907 from *Purpura lapillus,* and concluded that this cercaria is the larval stage of *Parorchis acanthus.* She also emphasized the close affinity of the genus *Parorchis* to the genus *Echinostomum* and expressed the belief that in the life history of *P. acanthus* there must be a secondary intermediate host, probably a bivalve mollusk, as is the case with *Echinostomum secundum* (Lebour, 1908). In a later paper, Lebour and Elmhirst (1922) reported the encystment of cercariæ of *Parorchis acanthus,* together with the cercariæ of *Echinostomum secundum* in the foot and mantle of *Cardium edule* and *Mytilus edulis.* No feeding experiments were made in determining the life history of *P. acanthus,* and conclusions were based entirely on morphological comparisons.

Methods and Observations

The present study consisted of controlled feeding experiments, since this is the only conclusive method of determining the life history. The oyster drill, *Urosalpinx cinereus,* was used exclusively as a source of cercariæ since the study was practically completed before it was discovered that *Thais* (*Purpura*) *lapillus* also served as host for this species. The infected snails were isolated in finger bowls. Cercariæ escaped from them in large numbers and encysted on the bottom of the bowl. These cysts were scraped off with a scalpel and concentrated by decantation. Attempts were made to infest terns, guinea pigs, white rats, and mice. Although the natural host of *P. avitus* is the herring gull, *Larus argentatus,* for the experiments, nestlings of two species of terns, the common tern, *Sterna hirundo,* and the roseate tern, *Sterna dougalli,* were employed since they were more easily obtained. The newly hatched nestlings were taken from a tern rookery on Big Weepecket Island, near Woods Hole, and were kept alive in the laboratory as long as possible. These birds, which had received no food while in the nest, were fed the deep flesh of mackerel and flounders, since trematode cysts are not as abundant in these tissues as in the skin and flesh near the surface of the body. Fishes showing any signs of being parasitized were not used for food. The nestlings were kept in the laboratory for two days, or until they began to take food readily, before infestation was attempted. They were then fed large numbers of cysts by means of a pipette. The first lot of 6 birds was fed cysts to determine whether infestation could be established. Five of these died at night and in all cases were so badly disintegrated by the following morning that the alimentary tract had almost completely disappeared. The sixth bird refused to eat on the tenth day and was killed. Twelve immature worms in various stages of development were recovered from the cloaca. The second lot consisted of eight birds. Six were fed cysts on the same day and two were kept as controls. The nestlings lived for varying lengths of time, two of the roseate species surviving as long as 15 days. Table I gives the results obtained with this series.

Table I

No.	Species	Fed Cysts	Autopsied	No. Worms
1	S. hirundo	July 11	July 16	11
2	" "	"	" 17	13
3	" "	control	" 17	none
4	" "	"	" 18	"
5	" "	July 11	" 20	3
* 6	" "	"	" 21	none
7	" dougalli	"	" 26	13
8	" "	"	" 26	28

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* This bird died during the night and was badly disintegrated when autopsied, which may explain the absence of worms.

It is seen that every bird which was fed cysts, and on which a favorable autopsy could be made, became infected, while the two controls were negative. The oldest worms were recovered from the two roseate terns which lived 15 days after infestation was established. Although not sexually mature, these specimens were sufficiently developed to be identified positively as *Parorchis avitus;* this identification has been confirmed by Professor Edwin Linton.

Further attempts at experimental infection were made, using mammals instead of birds. Four guinea pigs, nine rats and six mice were fed large numbers of encysted larvæ and were autopsied after varying lengths of time. No worms were recovered from any of these animals. Although the larvæ may possibly excyst in these mammals, it is evident that conditions are not suitable for establishing infection.

Stages in the Life Cycle

The sexually mature worms are viviparous and miracidia occur free in the terminal part of the uterus. Each miracidium contains a single, fully formed redia and there is no sporocyst generation in the life history. These stages were described by Linton (1914). The rediæ

(Figs. 1 and 2) and cercariæ (Fig. 3) were described by Stunkard and Shaw (1931). It should be recorded that in addition to *Urosalpinx cinereus, Thais* (*Purpura*) *lapillus* also has been found to serve as the intermediate host of *Parorchis avitus.* Of 108 specimens of *Thais lapillus* collected August 1st at Weepecket Island, 8 were found to be infected. The appearance of the parasitized gonad and digestive gland of this snail was similar to that reported for *Urosalpinx cinereus* (Stunkard and Shaw, 1931). Of 610 *Urosalpinx* examined during the summer of 1931, 25 were found to be infested. This determination is based on the emergence of cercariæ from the isolated snails. The infestation of *Thais,* however, was found by crushing and examining them.

Most of the cercariæ encyst within 48 hours after leaving the snail. Encystment may be accelerated by mechanical stimulation such as stirring or shaking. Concentrated solutions of vital dyes also induce cyst formation. Before encysting, the cercariæ attach themselves by means of the suckers to the bottom of the dish or to the slide as the case may be, and become comparatively quiet for a few moments. The cystogenous material is then exuded, enclosing the body in a viscous mass. This material adheres to the tail, pushing it off from the body as additional cystogenous material is extruded. The tail may remain attached to the cyst for some time or may break off as a result of its vigorous movement. Decaudation before encystment has been observed a few times but this is not the rule. In either case, the tail soon disintegrates. The cystogenous material is poured out very quickly and remains in a semi-liquid state for about a minute, as indicated by its yielding to the movement of the enclosed worm. In a few minutes, however, the cyst becomes hardened. The cyst wall (Figs. 4 and 5), which is flattened on the side of attachment, consists of two layers, an outer thick and brittle one, and an inner membrane which is thin and tough. The outer wall is often broken when the cysts are scraped from the bottom of the dish with a scalpel.

The larva becomes shrunken immediately if the inner cyst membrane is torn and the worm comes in contact with sea water. This interesting phenomenon seems to be due to the hypertonicity of the sea water, although it may be the result of mechanical injury. If the former is the case, encystment must involve an important change in the body of the worm which before encystment can live perfectly well in sea water. The cyst is fairly transparent and the larva can be clearly observed within it. The position of the larva in the cyst is shown in Figs. 4 and 5. The acetabulum is often forced to the side of the median plane because of its large size. A fluid fills the space between the larva and the cyst wall. It is clear and in some cases contains small granules which have probably escaped from the excretory system of the worm. The main excretory tubules with their concretions are easily observed through the cyst wall. The larva moves for several hours after encystment and then becomes quiescent. Movement may be induced by warming very slightly; it ceases upon cooling. The encysted worms may remain alive for an extended period if kept in water, but they do not withstand any considerable desiccation. They have been made to move two weeks after encystment; it is not known how long they can survive.

Specimens of *Mytilus edulis* were placed in finger bowls containing hundreds of cercariæ in order to determine whether the larvæ would encyst within any part of the mollusk. These experiments were all negative. In one case, three cysts were found *on the surface* of the foot and mantle, and a few others were seen in masses of mucus in the mantle cavity. In the same specimen, large numbers of cercariæ encysted on the outside of the shell, most of them near the incurrent siphon. Many of them struck the shell around the incurrent opening, adhered, and immediately encysted. The significance of these results will be brought out in the discussion.

Since birds could be infected by feeding the encysted worms, and since the larvæ encyst on any available object, it is apparent that a second intermediate or transfer host is not an essential stage in the life cycle of the parasite. It is clear that the final hosts are infected by accidentally ingesting the larvæ with food on which they have encysted. The worms excyst in the intestine of the bird and develop to maturity in the cloacal region. In autopsies of experimentally infested birds, a few worms were found just behind the intestinal ceca, but none in the ceca themselves. Since sexually mature worms were not obtained, the following description is based on specimens recovered 15 days after infection. These worms possess all of the characteristic adult structures except the distended gonoducts. They are white in color and are seen only with difficulty on the wall of the cloaca. It is very difficult to remove the worms from the surface of the cloacal epithelium on account of the powerful suction of the acetabulum. After removal to Ringer-Locke's solution, specimens have been kept alive for twelve hours before fixation. It is not known how much longer they would have survived. If not removed from the cloaca soon after the death of the host, however, they die and disintegrate rapidly along with the tissue to which they adhere. The intestine of a bird is frequently disintegrated 6 to 8 hours after its death, which explains the negative results in the first series of experiments.

When the worms are placed in Ringer-Locke's solution, they move actively for about an hour. This movement is very similar to that of the cercariæ. The worms, although flat and leaf-like, tend to lie on one side, *i.e.,* with the ventral surface in a vertical rather than a horizontal position, movement being effected by flexing and extending of the preacetabular region of the body. After this period of active movement during which the suckers are not used, the worms settle to the bottom of the dish, become attached by means of the suckers, and move in measuring worm fashion. This means of locomotion is more effective than the erratic dorso-ventral lashing of the forebody. Finally, however, the worms become quiescent on the bottom of the dish, remaining in some cases sufficiently still to be drawn with the camera lucida; when stimulated by moving the dish or touching them with a needle, they again swim actively for a time.

The morphology of *Parorchis avitus* was described by Linton (1914). Study of the present specimens confirms the observations of Linton and permits certain additions to the description of the species. The average measurements of thirteen 15-day worms stained and mounted are: length, 1.8 mm.; width of oral sucker, 0.236 mm.; width of pharynx, 0.1 mm.; and width of acetabulum 0.433 mm. The details of spination are shown in Fig. 6. There are about 68 spines in the collar. The papillæ, noted in the larvæ, still persist on the anterior end of the 15-day worm and are most clearly seen on a well extended but not flattened living specimen. The antero-ventral surface is the most heavily spined region of the body. The alternate rows of closely set spines extend backward in regular order to the genital ridge. Behind this region, there is a gradual thinning out on either side of and posterior to the acetabulum, with a few scattered spines extending to the extreme posterior end. The body is flattened dorsoventrally, especially in the postacetabular region, although with sexual maturity this portion will undoubtedly enlarge.

The excretory system, which is one of the most striking features of these specimens, differs markedly from the condition in the cercarial stage described by Stunkard and Shaw. In the cercaria there is an excretory vesicle at the caudal end of the body and collecting ducts pass forward on either side to the region between the pharynx and oral sucker where they turn posteriad. The recurrent ducts divide at the level of the intestinal bifurcation into anterior and posterior branches. Each of the anterior and posterior branches bears three groups of flame cells, with three cells in each group. In specimens removed from the cloaca of the tern the ascending collecting ducts of the cercaria have developed complicated series of evaginations which tend to form a reticulum and which ramify through the body of the worm. This ramification of the ascending ducts has not been observed in encysted larvæ and probably does not appear before excystment. A number of very small worms were recovered from the tern that was fed cysts intermittently. The youngest of these, which had probably been excysted not more than two or three days, exhibited to a considerable degree the complex ramification of the main excretory trunks. In general, the excretory system agrees with that of *P. acanthus* as described by Nicoll (1907 *b*). The excretory vesicle is a conspicuous, irregularly shaped, transparent sac at the posterior end of the body, and empties through a dorsal excretory pore (Fig. 6). The main lateral excretory trunks extend from the antero-lateral regions of the vesicle and branch in a very complicated manner. The rami extend in three general directions, laterad, mediad, and anteriad. The median branches either end blindly or anastomose with corresponding branches of the opposite trunk. The anterior branches are, for the most part, continuations of the main trunk which extend around either side of the acetabulum, anastomosing in some cases, and sending a number of sinuses into the region just above the acetabulum. The lateral branches extend laterally and somewhat ventrally, connecting with a more or less continuous row of large sinuses which extends along either side of the body. Tiny branches extend from the sinuses to the very edges of the body. The two rows of lateral sinuses extend posteriorly and in some cases fuse, making a connection behind and below the excretory vesicle. Anteriorly, the anterior sinuses decrease in size and send branches into the tissue just dorsal to the oral sucker. On each side, at the level between the oral sucker and

EXPLANATION OF PLATE

Fig. 1. A young redia with an immature cercaria and several germ balls (42 ×).

Fig. 2. An older redia containing only mature cercariae, one of which is seen escaping through the birth pore (42 ×).

Fig. 3. A mature cercaria (155 ×).

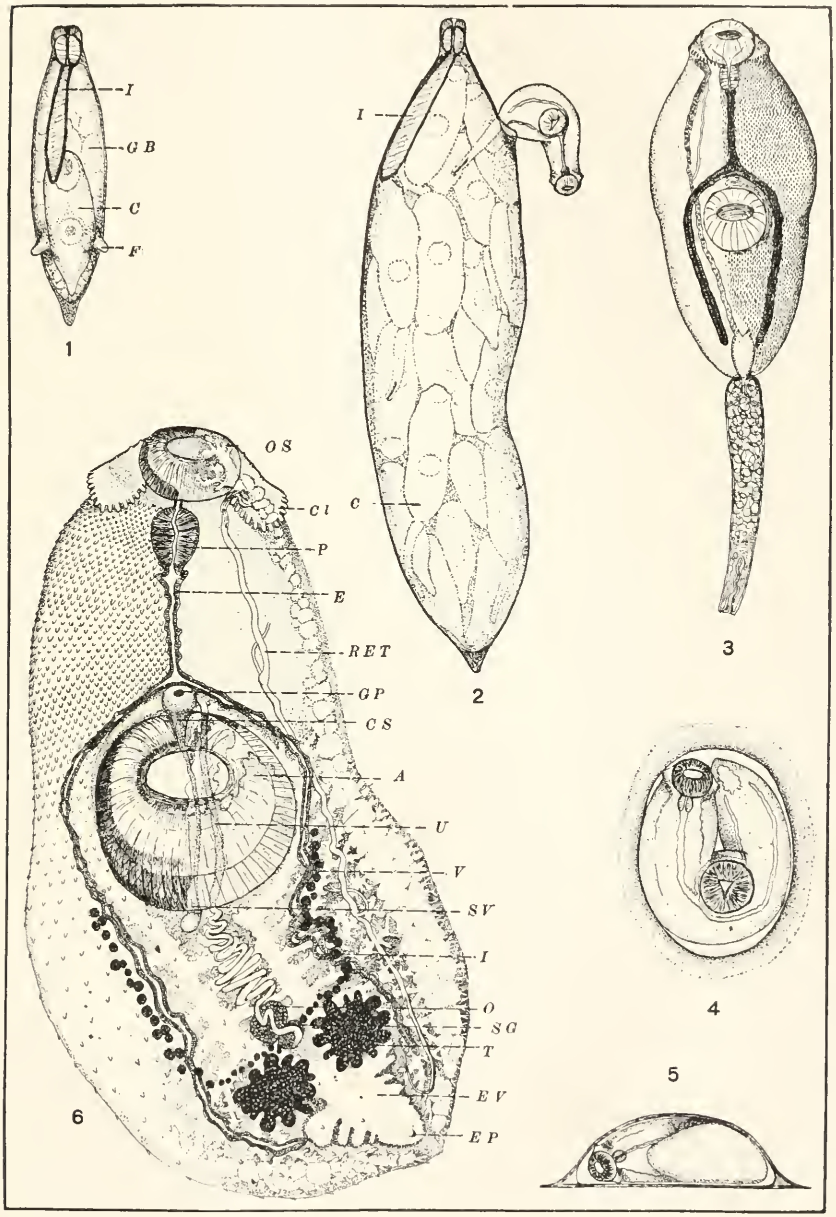
Fig. 4. An encysted cercaria, top view (132 ×).

Fig. 5. An encysted cercaria, side view (132 ×).

Fig. 6. Ventral view of a 15-day worm (60 ×).

LETTERING

A	Acetabulum
C	Cercaria
Cl	Collar
CS	Cirrus Sac
E	Esophagus
EP	Excretory Pore
EV	Excretory Vesicle
F	Foot
GB	Germ Ball
GP	Genital Pore
I	Intestine
O	Ovary
OS	Oral Sucker
RET	Recurrent Excretory Tubule
SG	Shell Gland
SV	Seminal Vesicle
T	Testis
U	Uterus
V	Vitellaria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pharynx, the recurrent excretory tubules extend inward and backward from the lateral sinuses, dividing at the level of the intestinal bifurcation into anterior and posterior branches, as described in the cercaria. It is seen, then, in the adult that the complex sinus system as a whole corresponds to the main lateral trunks of the cercaria and, consequently, serves to connect the recurrent excretory tubules with the vesicle. The main tubules are ciliated and the entire system, with the exception of the recurrent tubules and their branches, contains numerous concretions. The movement of these excretory bodies in the sinuses is of assistance in tracing the connections of the system. The excretory fluid is forced through the spaces by movements of the worm, the direction of the flow depending on the movement. After living worms have been kept in Ringer-Locke's solution for some time, the sinuses and bladder collapse, expelling clouds of granules and considerable amounts of fluid through the excretory pore. This makes it necessary to study the excretory system in freshly removed material, if the precise relations of the vesicle and sinuses are to be observed. The worm may live, however, for many hours after these structures have become indistinct. The preacetabular region of the body also becomes opaque long before the worm dies, making observations on the finer details of the excretory system in this region more difficult.

The reproductive system (Fig. 6) of the 15-day worm is not fully developed, although in a few cases motile spermatozoa were observed in the seminal vesicle, and one case of copulation was noted. The uterus begins as a simple duct which extends forward from the oötype. As development proceeds it forms lateral convolutions, a process which continues as the worm becomes more mature and large numbers of eggs are produced.

Discussion

Since the close affinity of *Parorchis* and the echinostomes is apparent from morphological comparisons, and since the cercariæ of *Echinostomum secundum* were found by Lebour (1908) to encyst in bivalve mollusks, she expected the same behavior on the part of cercariæ of *P. acanthus*. Several facts, however, seem to indicate that Lebour and Elmhirst's (1922) conclusions in regard to the encystment of cercariæ of *P. acanthus* (*C. purpuræ*) in the mussel, *Mytilus edulis*, may be in error. Close relationship may not necessarily imply identical modes of life history. Johnson (1920) has shown in the case of *Echinostomum revolutum*, which is cogeneric with *E. secundum*, the cercariæ encyst both in the snail bearing the rediæ, and in turbellarians. Johnson emphasized the lack of specificity in this regard. Furthermore, Lebour and Elmhirst's experiments were not adequately controlled. A criticism of their methods and results was made by Stunkard and Shaw (1931). The flattened side of the cyst, as figured by Lebour and Elmhirst, indicates that it had been formed on a surface over which it had spread somewhat before hardening, and not in the tissues of the foot or mantle. It is difficult to conceive the formation of a cyst of this shape within the soft tissue. The paucity of their positive results and the fact that their experiments were not sufficiently controlled, throw considerable doubt on the conclusions of Lebour and Elmhirst in regard to a second intermediate host for *P. acanthus.* Their negative results were explained by the assumption that the cercariæ were not ready for encystment, a certain amount of swimming being necessary before this process occurred.

In the present experiments, the cercariæ encysted quite readily on the shell of *Mytilus* but exhibited little or no tendency to do so on the foot or mantle. Johnson (1920) stated that larvæ of *Echinostomum revolutum,* encysted in the snail, were not killed by the death and disintegration of the host. The encysted cercariæ of *Parorchis avitus,* similarly, may not be adversely affected by the death and partial disintegration of organisms on which they encyst. The gulls, which are natural hosts of this trematode, are scavengers, feeding on dead material. It appears certain from observations made in the present study that the cercariæ, after leaving the snail, may encyst *on* almost anything with which they come in contact. The ease with which encystment can be induced by mechanical stimulation indicates that merely swimming against organisms, or even inanimate objects, may cause encystment. It has been shown that encystment and subsequent development in a secondary intermediate host are not necessary for the completion of the life history. The encystment of the cercariæ on anything that may become food for gulls provides their means of reaching the final host, and is the essential factor in completing the cycle.

A morphological comparison of *Parorchis acanthus* and *P. avitus* shows many similarities, particularly in regard to the excretory system, the reproductive organs, the scale-like spines of the collar and body, and the general body shape. A close comparison of the sizes of suckers and coils of the uterus is not possible at the present time since our specimens of *P. avitus* were not fully mature. Both the oral sucker and acetabulum increase rapidly in size after excystment, but the rate of increase is much greater in the case of the acetabulum, the ratio changing from about 1 : 1.4 in the cercaria to approximately 1 : 3 in the adult.

The similarities between the two species appear to be of generic significance, while present observations note additional specific differences between *P. acanthus* and *P. avitus.* The chief differences, compiled from the descriptions of Linton, of Lebour, and from the present study are: (1) the ratio of sucker diameters, which is 1 : 3 in *P.* avitus and 1 : 2 in *P. acanthus;* (2) the greater extent of uterine convolution in *P. avitus;* (3) *P. avitus* is viviparous whereas *P. acanthus* is oviparous; (4) the miracidia are very different in appearance; (5) the excretory system extends into the tail of the cercaria of *P. avitus,* whereas it does not enter the tail of *Cercaria purpuræ;* (6) the arrangement of the gland cells in the cercariæ is different; and (7) the larvæ of *P. avitus* do not encyst in the foot or mantle of *Mytilus edulis* as has been reported for *P. acanthus.*

SUMMARY

The life history of *Parorchis avitus* has been experimentally traced. The cercariæ occur in the marine snails, *Urosalpinx cinereus* and *Thais* (*Purpura*) *lapillus.* Adults have been obtained from the cloaca of the common tern, *Sterna hirundo,* and the roseate tern, *Sterna dougalli,* after feeding the young birds with encysted larvæ.

It has been shown that a specific secondary intermediate host is not essential for the completion of the life history; only a means of transference is necessary.

Additional morphological differences between *Parorchis avitus* and *Parorchis acanthus* are described.

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